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Resumen por los autores Libbie H. Hyman y Albert E. Galigher.

Demostración directa de la existencia de un gradiente metabólico
en los anélidos.

Por medio de la susceptibilidad y de los métodos eléctricos los autores han acumulado pruebas que indican claramente la existencia de un gradiente metabólico doble en los anélidos oligoquetos y poliquetos. Según estas pruebas, los extremos anterior y posterior poseen la mayor proporción metabólica, la cual decrece a partir de dichos extremos hacia las regiones medias; en la mayor parte de las formas el extremo posterior posee mayor proporción metabólica que el anterior. En el presente trabajo los autores dan a conocer determinaciones directas de la proporción de oxígeno consumido por unidad peso de trozos de las regiones anterior, media y posterior del oligoqueto *Lumbriculus inconstans* y de los poliquetos *Nereis virens* y *Nereis vexillosa*. Los resultados de estas determinaciones son que los trozos posteriores de estas especies consumen más oxígeno por unidad peso durante unidad tiempo, mientras que los trozos de la región anterior consumen menos y los de la media la menor cantidad. Los resultados, por consiguiente, confirman las conclusiones derivadas de los otros métodos y establecen de un modo indudable la existencia de un gradiente metabólico doble en estas formas.

Translation by José F. Nonidez
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DIRECT DEMONSTRATION OF THE EXISTENCE OF A METABOLIC GRADIENT IN ANNELIDS

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THREE FIGURES

INTRODUCTION

For a number of years evidence has been presented from this laboratory concerning the existence of metabolic gradients in organisms. This evidence has been obtained through the use of several methods. These may be summarized briefly as follows:¹

1. *Regeneration method.* In pieces cut from different levels of the axis of simple organisms the rate of regeneration and the kind of structure regenerated exhibit graded differences correlated with the level from which the pieces are taken. These differences in the regenerative phenomena at different levels cannot be due primarily to morphological factors, but are functional in nature, since they depend upon the size of the piece and are easily alterable by conditions whose action is chiefly quantitative.

2. *Direct susceptibility method.* Different levels of the axis of simple organisms exhibit a differential susceptibility to concentrations of toxic substances which will kill within a few hours. Since the death gradients of organisms in such solutions are the same for a large variety of toxic substances of widely different chemical constitution, these gradients cannot be due solely to the specific mode of action of these substances, but must depend rather upon a general quantitative gradation of some sort existing within the organism itself. The suscepti-

¹ For a more complete discussion of these methods together with references consult Child ('20).

bility method is also used to control morphogenesis during regeneration or development. For this purpose less concentrated solutions are employed which do not kill, but depress. This depression is differential; consequently, when regenerating pieces or eggs or embryos are exposed to the proper concentrations, morphological modifications result which are predictable and explicable on the basis of the susceptibility gradient. That differential susceptibility to toxic solutions is correlated with differences in metabolic rate is shown by the fact that conditions which are known to modify metabolic rate also modify susceptibility and in the same direction.

3. *Indirect susceptibility method.* The death gradient of organisms in dilute concentrations of toxic substances such that death occurs only after several days' exposure is exactly the opposite of the death gradient in quickly lethal solutions. This shows that the susceptibility results are not due to differential permeability, since in that case it would be impossible to account for the difference in the action of solutions of different concentration. The reversal of the gradients in dilute solutions is believed to be due to differential acclimation. Differential acclimation also accounts for certain modifications induced during regeneration and development exposure to the proper concentrations of toxic substances.

4. *Indophenol method.* When organisms are exposed to a mixture of dimethyl-*p*-phenylene-diamine and α -naphthol a blue precipitate of indophenol is produced. It is generally accepted that the formation of this precipitate depends upon the presence of oxidative processes. The depth of color produced along the axes of organisms through this reaction exhibits the same gradation as evidenced by the direct susceptibility and other methods. This method has been used only for embryonic stages and small forms.

5. *Potassium permanganate method.* When exposed to solutions of potassium permanganate organisms reduce it to manganese dioxide. The capacity to carry out this reduction exhibits the same gradation along the axis as appears by other methods.

6. *Electrical methods.* With the aid of a galvanometer it is found that permanent differences of potential exist along the axes of animals. These electrical gradients correspond to the susceptibility, staining, and reduction gradients, the regions of highest susceptibility, greatest staining, and reducing powers being electronegative (galvanometrically) to regions of lower susceptibility, staining, and reducing powers. The galvanotactic behavior of at least the simpler organisms is also in accord with the metabolic gradient conception.

The concordance of results obtained by these various methods indicates clearly enough the existence of a definite gradation along the axes of organisms. The nature of this gradation is naturally open to discussion. Our explanation that the gradation is primarily quantitative involving differences in the rate of fundamental metabolic reactions and in conditions associated with these is the only one that fits all of the facts at present known to us.

It would obviously be desirable, in order to establish the metabolic nature of the gradients beyond all reasonable doubt, to make quantitative tests of the rate of metabolism of different levels of the axis. To do so would appear to be a relatively simple matter, but in fact a number of complicating factors are involved. In the first place we must consider whether the isolation of pieces from different levels involves physiological changes in the pieces. We have definitely shown for *Planaria* that it does involve considerable metabolic increase, and that this increase is related quantitatively to the length of the pieces and the level of the body from which they are taken. To eliminate this factor it is necessary either to cut pieces so long that the stimulation of cutting is reduced to a minimum or to wait until the stimulation has disappeared, as happens after a number of hours. In the former case an organism of sufficient length would have to be selected; in the latter case one encounters changes due to the onset of regeneration. It is further necessary to select organisms which present neither structural nor special functional differences along their axes. The functional activity of some specific part may not accord with the general gradient, and

determinations of the total metabolism of such parts might therefore give results seemingly at variance with the gradient conception. The gradients of particular organs or structures are not necessarily the same as those of the organism in general; we know of many instances of such secondary gradients. Selection of the proper organism and elimination of these complicating factors are therefore essential before determinations of the total metabolism of isolated pieces possess any meaning.

On thinking over the organisms available for such experiments, it is obvious that they are few in number. Planarians are usable only under carefully controlled conditions. Under such control it has already been shown that the carbon-dioxide production at different levels of the anterior zoid corresponds with the gradient conception (Robbins and Child, '20), and similar experiments on the oxygen consumption are now in progress. Certain hydroids could probably be used, and experiments on them will be undertaken as soon as opportunity affords. The annelids are, however, by far the most suitable organisms for this kind of experimentation. Among the annelids there are many forms which are morphologically much the same throughout the greater part of the body in the sexually immature condition. They are, moreover, long enough to permit the cutting of pieces of the length necessary to eliminate the stimulation of section. As far as we have been able to determine, there are, in the non-sexual state, no special regions having a rate of activity different from that of the body in general. The susceptibility method has shown that the gradient of the digestive tract is the same as that of the body wall. By means of methylene blue the gradient of the nervous system has likewise been determined to be of the same character.² In these animals, then, there is every reason to predict that pieces cut from different levels of the body will show differences in respiratory rate which will accord with the susceptibility differ-

² In making a previous note on the use of methylene blue for demonstrating minor gradients (*Jour. Exp. Zool.*, vol. 24, p. 55), the senior author, being at that time ignorant of the fact, failed to credit Mr. John Wood MacArthur, formerly of this laboratory, with precedence in the use of this reagent, and takes this opportunity of correcting that omission.

ences. This prediction has been verified in the most satisfactory manner.

The experiments reported in this paper concern the rate of oxygen consumption per unit weight of pieces from anterior, middle and posterior levels of the polychaetes *Nereis virens* and *Nereis vexillosa*, and the oligochaete *Lumbriculus inconstans*. The experiments on *Nereis* were performed by the junior author at the Puget Sound Biological Station, Friday Harbor, Washington, in the summer of 1920. Those on *Lumbriculus* were performed by the senior author at the University of Chicago in the fall of 1920.

EXPERIMENTS ON LUMBRICULUS

1. *The susceptibility gradient of oligochaetes.* The susceptibility gradients of a number of oligochaetes including *Lumbriculus inconstans* were described in detail by the senior author in a former paper (Hyam, '16). It was found that in oligochaetes in general a double gradient is present—that is, the anterior and posterior ends are the most susceptible to toxic solutions and this susceptibility decreases toward the middle. The gradient in these forms may then be described as of the shape of the letter U, except that the two limbs of the U are of unequal length. In some species the anterior end is more susceptible than the posterior end, in which case the left-hand limb of the U would be longer, while in other species the posterior end is more susceptible, making the right-hand limb of the U longer.

In *Lumbriculus inconstans* the posterior end of the body is generally the most susceptible part. In some individuals the head is about equally susceptible, but usually is less so. The susceptibility decreases from both ends toward the middle of the worm, the least susceptible part being anterior to the middle, generally in about the second quarter of the worm's length. These relations are subject to considerable variation in different individuals. In the posterior part of the body there are further present in many individuals physiological indications of the existence of a second zooid. *Lumbriculus* reproduces asexually by autotomy. It may break in pieces at any level, but very commonly breaks

at a more or less definite level near the posterior end (see line x in fig. 2). When stimulated or placed under altered conditions, the worms are likely to snap off their posterior ends, which thereupon regenerate into complete individuals. The vast majority of worms taken in the field will be found regenerating their posterior ends. The place at which the fission occurs is in many individuals detectable by its increased susceptibility to toxic solutions. Whether it preexists before the worm is placed in the toxic solution or whether autotomy is initiated (but not completed or rendered visible) as a consequence of placing the worm in the solution is difficult to say. Regeneration experiments reported in the paper already referred to indicate that the region preexists. It should be emphasized that not all individuals give evidence of the existence of a breaking level.

In figure 1 are presented graphs of the death gradients of three individuals of *Lumbriculus inconstans* in solutions of potassium cyanide. In constructing such graphs the number of segments is plotted on the abscissa and the time of death on the ordinate. As the posterior segments are considerably shorter than the anterior ones the number of segments per unit of the cross-section paper has been increased at the posterior end. The anterior end of the animal is at the left, posterior end at the right.

The graphs illustrate the general character of the gradient in this species. This type of gradient is characteristic of annelids in general, of vertebrate embryos, and in all probability of all segmented animals, at least in embryonic stages. It is due to the method of growth of segmented animals by the formation of new segments in front of the anal segment. All three of the individuals show the presence of the zone of autotomy described above; in two of them this zone is well marked, while in the third it is only slightly in evidence.

This susceptibility gradient involves not only the body wall, but also the digestive tract and all visible systems. The transparency of this species enables one to see everything except the nephridia. Sex organs are completely wanting. If, then, the susceptibility gradient is in reality a metabolic gradient, it must follow that in this worm pieces cut from the posterior end must

consume the most oxygen, pieces from the anterior end less oxygen, and pieces cut slightly anterior to the middle the least oxygen. This was found to be the case.

2. *The electrical evidence.* To complete the evidence brief mention may be made of the electrical gradient and galvanotaxis of oligochaetes. It has been found that the anterior and posterior ends of oligochaetes are electronegative (galvanometrically)

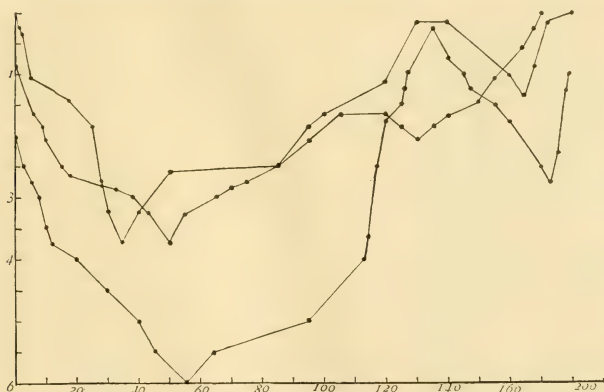


Fig. 1 Graph of the death gradients in cyanide of three individuals of *Lumbriculus inconstans*. Segments on the abscissa; time of death in hours on the ordinate, reckoned from the beginning of death, not from time in the killing solution. Heads of animals to left; posterior ends to right. The high peak near the posterior ends of two of the individuals is due to the presence of a zone of autotomy.

to the middle, and further that when placed in an electric current the worms bend into a general U-shape, with anterior and posterior ends directed toward the cathode and middle region directed toward the anode.³ These facts further indicate that the anterior and posterior ends of oligochaetes have a higher metabolic rate than the middle regions.

³ Cf. further Hyman ('18). Near the top of the second column on page 523 in this paper the statement that oligochaetes in a current 'travel to the anode' should read 'travel to the cathode.'

3. *Material and method.* The species used, *Lumbriculus inconstans* Smith, was obtained from pools in the woods near the village of Clarke, Indiana. These pools, filled with fallen leaves and moss, are the characteristic habitat of this species. The pools dry up in summer or fall, depending on their size, and when this occurs the worms pass into an encysted state, emerging when the pools become filled with water again in the spring. The best time to collect the worms in numbers is when the pool has dried down until only a small puddle of water is left in the lowest part. In this puddle the worms collect in great numbers and may be picked out by spreading out the debris from the pool on a dry spot. The worms used in these experiments came from three collections made on October 1st, 12th, and 15th, and from two different pools. They were kept in the laboratory in dishes of well-water filled with debris from their native pools and were used within three weeks from the time of collection.

The worms used were 40 to 60 mm. long and consisted of 150 to 200 segments. As many worms will be found regenerating the tips of the tails it was not possible to avoid entirely using such worms. Worms showing signs of regeneration elsewhere than the tip of the tail were discarded. Three pieces were cut out of each worm, as illustrated in figure 2. The head of the worm was cut off and discarded and the first piece then cut immediately behind the head. The second piece was cut immediately posterior to the first one. The third piece was cut near the posterior end, the extreme posterior end, including the regenerating tip if such were present, being discarded. The first two pieces were of equal length, being usually 3 to 5 mm. long and containing ten to twelve segments. The posterior piece was generally cut longer than the other two pieces, owing to the smaller diameter of the posterior end, and was about 8 to 10 mm. long, containing twenty to thirty segments. There was naturally some variation in the length of the pieces corresponding to the variation in the length of the worms from which the pieces were cut. These pieces are too long to be stimulated by the cutting except of course at the cut surfaces, where such stimulation always occurs. However, as each piece possesses two cut surfaces, this factor is equalized.

It may be emphasized that these pieces are as nearly alike morphologically as is possible to obtain in elongated animals. The digestive tract of *Lumbriculus* is not differentiated except

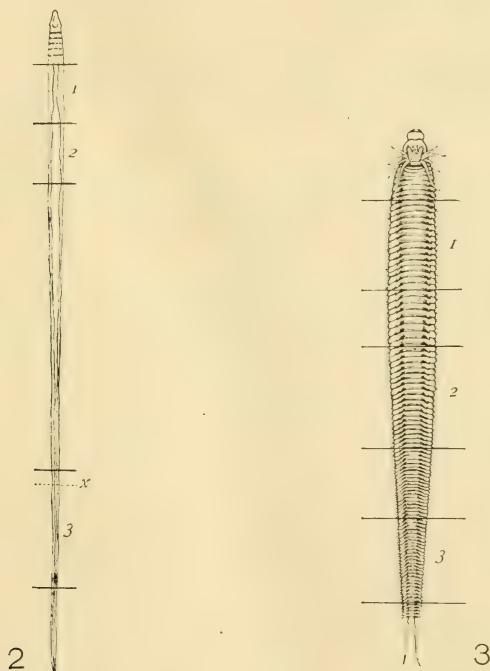


Fig. 2 *Lumbriculus inconstans*, showing the manner of cutting the three pieces whose oxygen consumption is given in table 1. *x* indicates zone of autotomy.

Fig. 3 *Nereis virens*, showing the manner of cutting the three pieces whose oxygen consumption is given in tables 2 and 3.

in the head region, where it forms a pharynx; but this, as, already stated, is cut off and discarded. There are no reproductive organs. The circulatory system forms a loop with the sacs characteristic of the lumbriculids in each segment. The only differ-

ences concern the relative proportions and sizes of the parts of the animal at different levels. Even such differences are practically nil between the first two pieces, as the diameter of body and digestive tract and length of segments are much the same throughout the anterior half of the worm.

By reference to the graphs in figure 1, it will be seen that pieces 1 and 2 are cut from the descending part of the gradient and that piece 3 generally includes the zone of autotomy when present. The lowest part of the gradient of *Lumbriculus* falls between the thirtieth and sixtieth segments. In order to avoid with certainty the ascending gradient of the posterior two-thirds of the body, the second pieces were cut well in the anterior part of this region of lowest rate. It is of course impossible to know the details of the gradient in each individual worm, and hence considerable variation in the total oxygen consumption per unit weight in different sets of pieces may be expected. It is nevertheless certain that the oxygen consumption will be correlated with the levels from which the pieces are taken.

As *Lumbriculus* is rather small it was necessary to cut a considerable number of pieces for each experiment. A count of the number of pieces was not made, but the number was probably in the neighborhood of two hundred. All of the pieces from each level were placed in a 500 cc. Erlenmeyer flask. This was filled air tight with water, of which a sample was taken for analysis. The flasks containing the pieces were then allowed to stand at constant temperature for twenty-four hours. In some experiments the pieces were cut in the morning and the experiment run from the afternoon of one day to the same time of the following day. In other cases the pieces were prepared late in the afternoon and the experiment begun the next morning. The second procedure was planned to eliminate any stimulation due to section if such might be present, but gave the same general results as the first plan. After the twenty-four-hour interval a sample of water was drawn from each of the experimental flasks and its oxygen content determined. The oxygen content was analyzed by Winkler's method. Further details will be found in previous publications (Hyman, '19). The pieces of worms

were then collected in a funnel of filter-paper, dried on filter-paper, and weighed. The weights of such sets of pieces varied from 100 to 200 mgm. in various experiments. The sets of pieces from the three levels were of course tested simultaneously in each experiment and were handled in the same way throughout. There was some movement among the pieces, particularly at the beginning of the experiments. As far as could be ascertained the amount of movement did not differ in pieces of different level.

TABLE 1

Showing the rate of oxygen consumption of pieces from different levels of Lumbriculus inconstans, the pieces taken as illustrated in figure 2 and tested simultaneously. Temperatures ± 1

NUMBER OF EXPERIMENT	TEMPERATURE	PIECE 1 (ANTERIOR)	PIECE 2 (MIDDLE)	PIECE 3 (POSTERIOR)
		Cubic centimeters of oxygen consumed per gram per twenty-four hours		
	°C.			
1	19.5	2.53	2.35	2.89
2	19.5	4.35	3.70	4.46
3	19.0	3.93	3.74	4.35
4	19.0	3.98	3.83	5.79
5	18.0	Lost	4.21	4.81
6	17.5	3.25	2.70	4.66
7	19.5	5.71	3.34	6.58
8	19.5	4.21	3.32	7.07
9	19.0	4.35	3.52	6.26
10	19.0	4.99	3.81	6.86
11	19.0	5.15	4.45	6.19
12	19.0	3.01	4.11	7.76
13	19.0	5.17	3.32	5.17

4. *Experimental results.* Thirteen experiments were performed, the final results of which are presented in table 1. The results are calculated in terms of cubic centimeters of oxygen consumed by one gram in twenty-four hours.

In all of the experiments except no. 12, the anterior pieces consume more oxygen per unit weight per unit time than the middle pieces. The reversal of this result in experiment 12 may reasonably be regarded as due to some error in the course of the experiment, probably a mixing of the sample bottles. In all of

the experiments the posterior pieces consume more oxygen than the middle pieces and in the majority of cases they also consume more than the anterior pieces. In experiment 13 the oxygen consumption of the posterior pieces is the same as that of the anterior pieces, while in experiment 2 it is but slightly larger than the latter; but in most cases the posterior pieces have a markedly higher rate of respiration than the other pieces. The differences in the oxygen consumption in different experiments may be assigned to variations in the animals themselves.

The results on the oxygen consumption at different levels are therefore in complete accord with the susceptibility gradients, as shown in figure 1. The existence of metabolic gradient in this annelid may be regarded as demonstrated.

EXPERIMENTS ON NEREIS

1. *The susceptibility gradient of polychaetes.* Owing to the opacity and toughness of the majority of polychaetes, it has not been possible to apply the susceptibility method to them except in the case of the family Syllidae. In this family the worms are of small size and sufficiently delicate and transparent to permit observations on the death gradient. In the Syllid *Autolytus cornutus* the senior author found the gradient to be like that already described for oligochaetes (Hyman, '16, p. 118). There are further at hand the observations of Cwiklitzer, ('05) on the death gradient of *Ophyotrocha puerilis*, one of the Eunicidae. In order to study the regenerative capacity of this species, Cwiklitzer cut off very small portions of the head and noted in many of the operated individuals the occurrence of disintegration processes. These changes began first at the posterior end, then at the head, or at both anterior and posterior ends simultaneously, and proceeded from both ends toward the middle. In *Nereis* kept in the laboratory the senior author has noted in several cases the death of the posterior region, beginning at the anal segment and proceeding forward. These scattered observations indicate that the susceptibility gradient of polychaetes is similar to that of the oligochaetes.

2. *The electrical gradient.* The electrical gradient of freshly collected *Nereis* and its galvanotactic reaction are the same as those already described for oligochaetes. The anterior and posterior ends are electronegative (galvanometrically) to the middle region. When placed in an electric current, the worms bend into a U-form, anterior and posterior ends directed toward the cathode and middle toward the anode.

From these lines of evidence it may be expected that the oxygen consumption of different levels will vary in the manner already described for *Lumbriculus*. Posterior levels should consume the most oxygen, anterior less, and middle pieces the least. Experiment proves that such is the case.

3. *Material and method.* Two species were employed, *Nereis virens* Sars and *Nereis vexillosa* Grube. The former species was collected from the docks at the town of Friday Harbor; in such locations the worms are found among the *Mytilus*, tunicates, and similar forms which cover the piles. A few individuals of this species were also collected along the shores. The worms used were about 150 mm. long. *Nereis vexillosa* was obtained by digging in gravelly and sandy shores at low tide. The individuals used in the experiments were from 160 to 180 mm. in length. Sexually mature individuals were discarded. The worms were used as soon as possible after collection—from half an hour to three or four hours in nearly all cases. In two cases, experiments 6 and 7, table 3, the worms were kept about twenty hours before being utilized.

In preparing the worms for the experiments, the first 25 mm. and the last 10 to 15 mm. of the body were cut off and discarded. Three pieces were then cut from the body of the worm, short pieces being discarded between the first and second and second and third pieces. The way in which the pieces were taken from the worm is shown in figure 3. The pieces were 30 to 40 mm. in length. In each experiment several worms were employed, generally six or seven, once five, and twice twelve. After cutting, the pieces were placed in finger-bowls and washed in several changes of sea-water until bleeding had ceased and the cut ends contracted. All of the pieces of each level were placed to-

gether in a wide-mouthed bottle and their rate of oxygen consumption was then determined as already described for *Lumbriculus*. The tests ran from one to two hours. The pieces were then dried on filter-paper and weighed. Six or seven such pieces weigh from 2 to 10 grams; anterior and middle pieces weigh more than posterior pieces.

It may be pointed out that such pieces from different levels are nearly identical morphologically. The only differentiated part of the digestive tract in *Nereis* is at the anterior end, and this part of the body was discarded. The posterior end with its terminal cirri was likewise discarded. The only differences among such pieces are differences in the proportions of parts, as in the parapodia, which are largest in the middle regions of the body. The middle pieces probably have the advantage of greater surface of exposure, but, as will be seen, they nevertheless respire the least. It should also be stated that there was very little movement among these pieces.

4. *Experimental results.* The experiments on *Nereis virens* are recorded in table 2; those on *Nereis vexillosa* in table 3. Only the final calculations of the cubic centimeters of oxygen consumed per gram per hour are given. In the first two experiments on *Nereis virens*, the posterior pieces were not tested.

Examination of these tables shows that anterior and posterior pieces of *Nereis* invariably consume more oxygen per unit weight per unit time than middle pieces. Further, in nearly all cases the posterior pieces have a higher rate of oxygen consumption than the anterior pieces. There is one exception to this, experiment 5 in table 3, where the oxygen consumption of the posterior pieces is below that of the anterior pieces. In experiments 5 and 7, table 2, the posterior pieces are but slightly in advance of the anterior pieces, but in the other experiments the difference is marked. These variations in the experimental results are no doubt due to physiological differences in the individuals employed.

The existence of a metabolic gradient along the axis of *Nereis* may therefore be regarded as demonstrated. This gradient is identical with the susceptibility and electrical gradients.

TABLE 2

Showing the rate of oxygen consumption of pieces from different levels of Nereis virens, the pieces taken as illustrated in figure 3, and tested simultaneously

NUMBER OF EXPERIMENT	TEMPERATURE	PIECE 1 (ANTERIOR)	PIECE 2 (MIDDLE)	PIECE 3 (POSTERIOR)
		Cubic centimeters of oxygen consumed per gram per hour		
	°C.			
1	10.5	0.053	0.032	
2	10.0	0.042	0.035	
3	10.5	0.036	0.030	0.051
4	10.2	0.047	0.036	0.059
5	12.0	0.062	0.040	0.063
6	9.0	0.046	0.034	0.058
7	12.0	0.054	0.037	0.057
8	10.7	0.039	0.031	0.053
9	10.5	0.053	0.033	0.061

TABLE 3

Same for Nereis virens

NUMBER OF EXPERIMENT	TEMPERATURE	PIECE 1 (ANTERIOR)	PIECE 2 (MIDDLE)	PIECE 3 (POSTERIOR)
		Cubic centimeters of oxygen consumed per gram per hour		
	°C.			
1	9.0	0.061	0.039	0.079
2	9.5	0.040	0.034	0.055
3	10.0	0.047	0.033	Lost
4	12.5	0.078	0.060	0.094
5	10.0	0.062	0.038	0.050
6	13.0	0.057	0.043	0.083
7	13.0	0.040	0.035	0.074
8	10.0	0.059	0.041	0.081
9	10.5	0.074	0.058	0.089
10	9.5	0.041	0.036	0.063

SUMMARY

By means of the susceptibility and electrical methods, evidence has been accumulated which clearly indicates the existence of a double metabolic gradient in the oligochaete and polychaete annelids. According to this evidence, the anterior and posterior ends possess the highest metabolic rate, and from these ends the rate decreases toward the middle regions; in the majority of forms the posterior end has a higher metabolic rate than the anterior end.

In this paper are presented direct determinations of the rate of oxygen consumption per unit weight of pieces from anterior, middle, and posterior regions of the oligochaete *Lumbriculus inconstans* and the polychaetes *Nereis virens* and *Nereis vexillosa*. The results of these determinations are that the posterior pieces of these species consume the most oxygen per unit weight per unit time, the anterior pieces less, and the middle pieces least.

The results therefore confirm the conclusions drawn by the other methods and establish beyond reasonable doubt the existence of a metabolic gradient in these forms.

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Resumen por el autor, Chikanosuke Ogawa.

Experimentos sobre la orientación de la vesícula auditiva en las larvas de los anfibios.

Las vesículas auditivas de los anfibios giran hasta recobrar la posición normal cuando se las invierte, conforme descubrió primeramente Streeter. Esta rotación puede tener lugar en un estado en el cual la diferenciación de la vesícula no se ha llevado a cabo todavía. La vesícula auditiva de Rana puede transplantarse en *Amblystoma*. También tiene lugar la rotación en la vesícula de Rana transplantada en posición invertida en *Amblystoma* y viceversa. Si la vesícula se hace girar 180 grados sobre su eje transversal la rotación no se lleva a cabo en algunos casos, a causa, probablemente, de la fusión de la piel con ella. Es difícil suponer como causa de la rotación a cualquier factor diferente de la quimotaxia que actúa entre la vesícula y el medio que la rodea.

Translation by José F. Nonidez
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EXPERIMENTS ON THE ORIENTATION OF THE EAR VESICLE IN AMPHIBIAN LARVAE

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TWENTY-FOUR FIGURES

Several papers have been published by Streeter ('06, '07, '14) on the remarkable phenomenon of automatic rotation of ear vesicles to their normal position after having been inverted, a fact which he first discovered in experimental studies on the tadpole. He found that when the ear vesicle of a larva of *Rana pipiens* or *Rana sylvatica* was removed and replaced in the same pocket in the reverse position (inside out), at the end of two weeks the operated vesicle had developed in its normal position. It might well be assumed that the vesicle had simply slipped back into its original position, since the vesicle, and any additional fragment of mesoderm, would exactly fit the pocket from which they were taken. In order to test this possibility he removed a vesicle from one specimen and transplanted it into the emptied ear pocket of another. This procedure was followed by the same results as the first. Further experiments demonstrated that the cells constituting the ear vesicles become specialized very early; moreover, that fragments of a vesicle may develop independently of the rest of the structure. Streeter concluded, therefore, that the posture of the membranous labyrinth is determined by some influence which interacts between the labyrinth and its environment. This interaction may be explained on the basis of one of three possibilities: 1) It may be due to the intrinsic motility of the vesicle itself, such as is seen in the healing of skin wounds of young larvae; 2) it may be that the nerve and ganglion mass serve to draw the vesicle into its proper position; 3) compression from the surrounding organs may mechanically bring the vesicle back to its normal position.

Spemann ('10) worked independently on a similar problem. He took out the ear cup of *Rana esculenta* larvae in a very early stage and replaced it in an inverted position on its transverse axis. His conclusions are just the contrary of those of Streeter, as he found that the ear vesicle continued to develop without returning to its original posture. Streeter is inclined to seek an explanation for this discrepancy in a difference in operation methods. The present paper is designed to throw further light on this and certain other related problems: 1) When does postural readjustment of the ear vesicles take place? Do the vesicles slip back, as it were, into their normal position immediately after the operation? Do they rotate before or coincidentally with differentiation of the various structures of the ear? 2) Does such rotation occur also in ear vesicles transplanted from one specimen to another of a different order? 3) What are the results when inversion is made on the transverse axis by Streeter's method?

I wish to extend my heartiest thanks to Dr. G. L. Streeter for his constant interest while this work was in progress, and I also take pleasure in acknowledging my indebtedness to him for many valuable suggestions, as well as for the use of his preparations of tadpoles operated upon and preserved at very early stages (from fifteen minutes to five and one-half hours after operation).

MATERIAL AND METHODS

For a study of the first and third problems I made use of *Rana palustris* larvae; for the second, *Amblystoma* and *Rana*. In order to prolong the period for operations, I placed the freshly collected eggs in a refrigerator, taking them out as needed. The method of operation was that described by Streeter ('14). Two larvae were placed side by side under a binocular microscope. With two embroidery needles an incision was made through the ectoderm of one of the specimens, over the site of the ear vesicle. With the needles the vesicle was loosened from its pocket and cast away. The vesicle of the second specimen was uncovered in a similar manner and slipped into the empty pocket

of the first, being placed so that its lateral surface would lie against the brain. The operation was performed at the end of the non-motile stage. It may be mentioned here that a slight inequality in the development of the larvae at the time of operation is almost unavoidable. The movement of the larvae at the time of operation, which is a source of annoyance to the operator, may be easily checked by placing a rusted needle in front of them. Only the right side of the animal was used for operation. For a study of the first problem, the specimens (*Rana palustris*) were fixed at various periods after operation, from fifteen minutes to thirty-four hours. For the second, I transplanted the ear vesicle of *Amblystoma* to *Rana*, and vice versa, in reversed position in the emptied ear pocket. Fixation was done two weeks after operation. In the third, the ear vesicles were taken out and put back in the same pocket upside down, without changing the posture in regard to the vertical axis. In this case transplantation was not performed, because there exists no characteristic to denote the upper or lower side of the vesicle, and therefore mistakes might easily occur when a vesicle is carried from one specimen to another.

Mueller's fluid was used exclusively for fixing purposes. The specimens were imbedded in paraffin and cut 10μ thick. In the first study all sections containing the operated ear vesicles were drawn serially at a magnification of 100 diameters. Recourse to these drawings greatly facilitated the study of the experiments.

RESULTS OF EXPERIMENTS

All of the larvae used were in an early stage of development when the ear vesicles present no sign of definite differentiation—a condition which makes the determination of their position difficult. The criteria necessary to decide this question constitute an important consideration.

In very early stages of normal development the ear shows as an invaginated cup derived from the deeper layer of the skin. In the next stage the cup is pinched off, its edges close in, and finally a complete vesicle is formed. The medial and ventral parts of its wall consist of high columnar cells, the nuclei of which

are situated at about the middle of the cell body. The lateral wall is thinner, being formed by a single layer of cuboidal or low columnar cells. By stripping off the skin the vesicle is usually broken open in the fused area. Therefore, when the inverted vesicle is found unclosed, as is generally the case a short time after the operation, the open side should indicate the lateral orientation in the original posture. There is, however, no clue by which to distinguish the upper from the lower side.

The vesicle increases gradually in size, more so in its vertical diameter, and presents an oval or pear-like form. The wall is thickest at its ventral side and, as stated above, is formed of high columnar cells, while the side walls, consisting of cuboidal cells, are always thinner. In this stage the lateral wall is still thinner than the medial. The upper dorsal part of the vesicle, from which later the endolymphatic appendage develops, is composed of somewhat higher cells. These constitute characteristics which greatly facilitate the orientation of the operated vesicle and serve as important criteria. As development proceeds, the vesicle wall increases in thickness especially in the ventromedial part. The relative difference in thickness between the medial and lateral walls diminishes gradually, thus reducing its value as a guide to the position of the vesicle. Sometimes the medial wall may even be thinner than the lateral wall. Besides these characteristics, the close connection between the acousticofacial ganglion and the medial wall affords a helpful clue to the position of the vesicle.

The above description concerns typical, normal development. In exceptional cases, however, deviations from this course might easily occur; for instance, in the early stages there may be little or no difference in thickness between the medial and lateral walls. Moreover, in judging the position of the vesicle, several conditions must be kept in mind. Since an ear vesicle was taken from one specimen and put into another, it is to be expected that there would be some slight differences between the vesicles of the normal side and the transplanted one. Furthermore, when the vesicles are transplanted they must first of all adapt themselves to the new environment. For these reasons the

transplanted vesicle is not exactly comparable to that of the normal side. The greatest obstacle, however, is deviation from the normal development in the transplanted vesicles, especially in regard to form and the relative thickness of the walls. In some cases the vesicles are quite round instead of oval in shape, and the walls retain a uniform thickness.

For the sake of brevity, the results of my study are herewith given in the form of a table (table 1). By '*complete rotation*' is meant that the ear vesicle, which had been transplanted in an inverted posture, had turned back and regained its normal

TABLE 1

TIME AFTER OPERATION	COMPLETE ROTATION	HALF ROTATION	NO ROTATION	EFFICIENT CRITERIA LACKING	NO VESICLE	OTHER CIRCUM- STANCES	NUMBER OF EXPERI- MENTS
15-30 min.	—	—	2	—	—	1	3
3 hrs.	—	—	2	—	—	1	3
4 hrs.	1	—	—	2	—	1	4
5½ hrs.	1	2	2	—	—	—	5
16 hrs.	6	—	2	3	1	—	12
18 hrs.	—	—	3	5	—	—	8
20 hrs.	2	—	—	3	1	1	7
22 hrs.	2	—	1	3	—	1	7
24 hrs.	5	3	1	3	2	3	17
26 hrs.	1	—	3	4	1	1	10
28 hrs.	4	—	1	2	1	1	9
30 hrs.	5	—	3	—	—	—	8
34 hrs.	5	—	—	2	—	1	8

position. '*Half rotation*' implies that the vesicle rotated to some extent only. Differences in thickness of the vesicle walls, the general form of the vesicle, etc., are considered under '*Efficient criteria.*' '*No vesicle*' covers the case in which the vesicle slipped out from the transplanted area and hence was lost. By '*Other circumstances*' are designated those factors which made further study of the specimen impossible, such as improper treatment of the material, disassociation of the vesicle, etc.

As shown in table 1, fifteen specimens were examined at various intervals, from fifteen minutes to five and one-half hours after the operation. The ear vesicles were found still

open in five and closed in seven cases. The vesicles seemed to make no rotation in the first three hours; some of them turned back to the normal position within five to five and a half hours. These experiments prove that the wound-healing process has nothing to do with the rotation of the vesicles, because the latter still remained in their position after the wound had healed completely. In other words, mechanical slipping back of the vesicle due to the healing process, cannot be considered as the cause of rotation.

No examination was carried on between five and a half and sixteen hours after operation. On examining the specimens from sixteen to thirty-four hours after operation, it was found that rotation was going on during these stages. Thus it was also demonstrated that rotation can occur in a stage in which differentiation of the semicircular canals, endolymphatic sac, and other parts of the vesicle has not yet taken place and the ear is represented merely by a round or oval vesicle, indicating only some differentiation in epithelium. The number of rotated vesicles in each hour did not show a constant increase in regard to lapse of time, which was contrary to expectation. This may be explained by the fact that in quite a number of the cases there was not sufficient evidence by which to judge whether or not the vesicles had rotated. Therefore, if these uncertain cases were included, the total number and proportion of rotated and non-rotated vesicles would undergo some change.

Individual variation also may play a great part in rotation. If specimens are studied in later stages, at a period when the vesicles begin to show further differentiation and therefore furnish more efficient criteria, we shall then be able to say with some degree of certainty that the vesicles completed rotation at a given time. I regret that, for this year, I have lost the opportunity of making such a study.

*Protocols of experiments**Examination 15 to 30 minutes after operation.*

A. The ear vesicle is located in the area bounded by the brain and the endoderm, and is superficially embedded in the latter. The wound is still wide open laterally. The brain is quite intact. The vesicle is open toward the medial side. The wall is thick, indicating an early stage of development. A slight disassociation is shown in the lower part of the vesicle, otherwise it appears normal. No rotation has as yet occurred.

B I. On account of disassociation, no study could be made.

B II. The inverted ear vesicle seems well fitted to its new position. The skin has not yet entirely healed over the wound. The opening of the vesicle turns medially and slightly dorsally. No rotation as yet.

Examination 3 hours after operation.

A. The vesicle is already closed. It cannot be determined from its appearance whether the two edges participated equally in the closure or whether one of them took a predominant part. The lateral wall of the vesicle is about twice as thick as the medial and its outer surface is convex, while the medial wall is rather flat. The skin has already healed over and is in contact with the lateral wall. Normally, the medial wall should be thick and the lateral wall thin in the early stages of development; since in this vesicle the order is reversed, it must be assumed that the vesicle is still in an inverted position.

B (fig. 1). This case presents about the same picture as B II (examination 15 to 30 minutes after operation). The vesicle is open toward the brain. The skin has not yet healed over the wound. No rotation.

C. Sections destroyed.

Examination 4 hours after operation.

A. Vesicle already closed. The lateral wall is flat, the medial wall convex, and the two are of equal thickness. It is difficult to determine the position in this case, but judging by the general shape of the vesicle, it might be said to be normally situated.

B. Vesicle already closed. Because of the uniform thickness of the vesicle wall, the position is difficult to make out.

C. Vesicle closed. No efficient criteria can be obtained.

D. Sections destroyed.

Examination 5½ hours after operation.

A. The lateral wall of the vesicle is convex, the medial wall flat. The latter shows the mark of a healed wound. Most probably this vesicle is still in its abnormal position.

B. The vesicle has not closed, the opening faces medially. No rotation.

C. Vesicle closed. The dorsomedial wall is less thick and may with certainty be taken for the original lateral wall. The vesicle has turned half-way.

D. The lateral wall of the vesicle is thinner than the medial wall and the arrangement of the cells here is irregular, suggesting the healed trace of the vesicle. Rotation is complete.

E (fig. 2). Vesicle not quite closed as yet and its opening turns ventrally. The dorsomedial wall is thick. The vesicle has rotated half-way.

F. Determination difficult in this case, owing to disassociation.

Examination 16 hours after operation.

A I. The transplanted ear vesicle is somewhat oval in shape. The thinnest part of the wall faces toward the brain, i.e., dorsomedially, while the thickest part turns ventromedially. The cells composing the thin wall are cuboidal in form; the thick wall consists of high columnar cells. The cell layer is everywhere simple. Close to the medial wall there is a group of cells which probably represents a ganglion. In this case, if the vesicle be regarded as in an earlier stage of development, it might be said to have remained in its inverted position, since at this time the lateral wall is usually thin; but to judge from the development of the macula the vesicle must be older. Therefore, taking the position of the macula as a criterion, the vesicle has rotated back to its normal position. The presence of the ganglion at the medial side of the vesicle bears out this conclusion.

A II. Due to injury at the time of operation, the brain is a little deformed and in close contact with the wall of the ear vesicle, probably, however, without any actual fusion. The wall is thickest ventromedially. Some parts of the wall are rich in yolk granules. The skin has only partly healed over the vesicle. The whole appearance of the specimen deviates from the norm and does not warrant a definite decision as to the position of the vesicle.

A III. The whole shape is slightly abnormal. The wall is of uniform thickness throughout. There are not enough criteria by which to judge position.

B I (fig. 3). The vesicle is somewhat small and in section presents an oval form. The wall is thick. The thickest part turns dorso-medially, the thinnest ventrolaterally. The vesicle is evidently retarded in its development. Since the position of the thickest wall and that of the ganglion are reversed, the vesicle evidently has not rotated.

B II. No vesicle was found.

B III. The vesicle is of oval type. The axis runs vertically, with a slight lateral inclination. The thinnest part turns ventrolaterally and the thick part ventromedially. A group of round cells, containing yolk granules and pigment, is present in the cavity. This cell mass may have been taken in from the outside when the vesicle closed. Rotation is nearly complete.

C I. The vesicle is round and small. Because of the uniform thickness of the wall, it is difficult to ascertain the degree of rotation.

C II. The vesicle is small and round in section, but by measuring the serial sections it is found to be elongated in its sagittal plane, being especially narrow in its posterior part. A ganglion is closely attached to the ventral side. Although the wall is of uniform thickness, the general shape and position of the ganglion would certainly indicate that the vesicle is still in the reversed position.

C III. Vesicle small. The wall is thick ventrally and composed of high columnar cells. There is an especially thin part in the lateral wall, suggesting the closed part of the vesicle. Several round cells containing yolk granules are found in the cavity. Rotation is most probably complete.

D I. The vesicle is small and slightly oval. No significant difference in thickness of the wall is to be recognized, but the ganglion, which is closely attached to the medial wall, indicates the position of the vesicle. Rotation is complete.

D II (fig. 4). Vesicle is of medium size and in section oval. The ventromedial wall is thick, the dorsolateral wall thin. The general appearance gives every indication of a normally placed vesicle.

D III. Vesicle oval in form. The lateral wall is thinner than the medial; the wall is thickest ventromedially. Rotation is complete.

Examination 18 hours after operation.

A I. The vesicle contains a large cell mass in the lateral wall. The whole structure is pathological.

A II. The vesicle has three separate chambers. One is located dorso-anteriorly, the second ventroposteriorly, and the third and smallest, ventral to the first. The first two cavities are oval in shape. There are two possibilities which might account for the formation of such a structure: either the vesicle might have been severed in some way at operation or the original vesicle may not have been entirely removed, part of it remaining in position and fusing with the transplanted vesicle. The latter hypothesis, however, is rather doubtful, since we know from the experiments of Streeter ('09) that two vesicles do not fuse when placed in immediate contact. Since the structure is abnormal, the posture cannot be determined.

A III. The vesicle is small in size and oval in shape, the wall being of uniform thickness throughout. The ganglion is attached to the lateral side. The vesicle appears to be still in an inverted position.

B I. Vesicle is small. Ventral wall is thick. Position difficult to determine.

B II. The vesicle is too small to justify any conclusion.

B III. Vesicle small. The thinnest part of the wall turns medially. Probably no rotation has occurred.

C I. The cavity of the vesicle is small, the lateral wall thick. No rotation, or at most only partial rotation, is indicated.

C II. Pathological.

Examination 20 hours after operation.

A I. No vesicle found.

A II (fig. 5). The vesicle is large and of oval shape. The axis is directed mediolaterally with medial inclination. The ventral wall is thick. The ganglion is attached to the ventromedial wall. Thus rotation is in some measure complete.

A III. The cavity of the vesicle is divided into two. The whole structure is too abnormal to determine the position.

B I. The vesicle is small. The cavity is divided into several parts.

B II. Material incorrectly cut.

B III. Vesicle small and somewhat elongated in its sagittal axis. The wall is thick ventromedially. Rotation is complete.

B IV. Vesicle too small to justify conclusion.

Examination 22 hours after operation.

A I (fig. 6). The vesicle presents an oval shape in its mediolateral axis. The lateral wall is thick, the medial wall thin. A large cell group, probably the ganglion, is attached to the dorsal wall. No rotation.

A II. The vesicle appears oval in section with a slight vertical elongation. The ventral wall is thickest, especially in its medial part. Slightly caudal the ganglion is attached to the medial wall of the vesicle. No significant difference is recognizable between the medial and lateral wall. Rotation is complete.

A III. Judged by its general form, this vesicle appears to have completed rotation, but a definite criterion is lacking.

B I. The vesicle, oval in shape, is in its normal position. The dorsal part indicates the formation of the endolymphatic appendage. Rotation is complete.

B II. Series incomplete.

C I. Criteria lacking.

C II. The vesicle is divided into two parts, an upper smaller part and a lower larger one. The wall is thick ventrally. It is difficult to draw any conclusion as to its position.

Examination 24 hours after operation.

A I. The form and thickness of the wall afford no clue as to the position of the vesicle.

A II. This vesicle is entirely normal and corresponds to the normal side in every respect. Probably, through some mistake the original vesicle was not removed.

A III. Vesicle is oval. The wall is thick ventrally and in its lower medial part. The ganglion is attached to the dorsomedial part. Rotation is complete.

B I. The vesicle is round. The wall is thin laterally and thick medially. The ganglion is attached to the ventral wall. Rotation is half completed.

B II. Vesicle broken.

B III. The vesicle is round in section. The medial wall is thick, especially in the upper part. Rotation is half completed.

C I. The vesicle is nearly round in section. The dorsomedial wall is thick. Vesicle has rotated half-way.

C II. Sections destroyed.

C III. Vesicle too small for study.

D I. No criterion could be found in this case.

D II. No vesicle.

D III (fig. 7). The vesicle is small and round. The wall is thin laterally and distinctly thick ventromedially. Rotation is complete.

E I (fig. 8). The vesicle is oval with the axis inclined laterally. A part of the lateral wall is still open; this must be the original lateral side. The skin has healed thoroughly. Rotation complete.

E II (fig. 9). Vesical oval. The ventral wall is thick; lateral wall thin and flat. The whole appearance suggests complete rotation.

F I. Vesicle round; dorsomedial wall thick. The ganglion is attached to the ventromedial wall. Rotation probably is incomplete.

F II. Vesicle large and of oval shape. The lateral wall is flat. The whole appearance suggests complete rotation.

F III. No vesicle.

Examination 26 hours after operation.

A I. This vesicle presents an oval form. The wall is thick ventromedially. The ganglion is attached to the lateral wall. If the thickness of the wall is taken into account, the vesicle must have rotated, but the position of the ganglion speaks rather against such an assumption. Position is difficult to determine.

A II. The vesicle lacks the necessary criteria for decision.

A III. Vesicle round. The dorsal wall is thick, ventral wall thin. The ganglion is attached to the ventral wall. Rotation is not complete.

B I. The vesicle is large. The wall which turns cerebrally, is flat. This seems to have been due to pressure from the vessel which lies between the vesicle and the brain. The ganglion is attached to the ventral wall of the vesicle. Efficient criteria lacking.

B II. No vesicle.

C I. The vesicle is large. The dorsomedial wall is thin. One part of the dorsal wall is elongated upward. Position is difficult to determine.

C II. The wall is thin ventromedially and thick dorsally. The ganglion is attached to the ventral wall. No rotation.

C III. The vesicle is too small to admit of a definite decision.

D I (fig. 10). The vesicle is slightly oval. The wall is thick ventrally. The whole appearance suggests quite normal position. Rotation is complete.

D II (fig. 11). The vesicle is round. The dorsal and medial walls are thick, while the ventral wall is thin. Rotation not yet completed.

Examination 28 hours after operation.

A I. Specimen incorrectly cut and no vesicle was found.

A II. No vesicle found.

A III (fig. 12). The vesicle presents an oval shape. The wall is thick ventrally and thin laterally. In the ventro-anterior part the cavity is divided into two parts. The vesicle shows a dorsal prolongation which suggests formation of the endolymphatic appendage. Rotation is complete.

B I. The vesicle is round; the ventral wall thick. Position difficult to determine.

B II. Vesicle slightly oval. The wall is thick ventromedially. Rotation is complete.

B III (fig. 13). The vesicle is quite oval, the ventromedial wall thick. The ganglion is attached to the medial wall. Rotation is complete.

C I. The wall of the vesicle is thick ventromedially and thin laterally. The ganglion is attached to the medial wall. Rotation is complete.

C II. The vesicle is slightly oval. The ventral wall is thick. No accurate criteria are obtainable.

C III. Vesicle round. Lateral wall thick. Probably no rotation has occurred.

Examination 30 hours after operation.

A I. Vesicle oval in form: ventromedial wall thick. Rotation is probably complete.

A II. (fig. 14). Vesicle large and oval. The wall is thick dorsally. The ganglion is attached to the lateral wall. Rotation has not yet occurred.

A III. The vesicle is relatively small and round. The wall is thick dorsomedially. No rotation has occurred.

B I. Vesicle oval in form. The lateral wall is thin and slightly flat. Rotation is probably complete.

B II. Vesicle round. Ventromedial wall fairly thick. Rotation complete.

B III. Vesicle round. The difference in thickness of the walls is insignificant. The ganglion is attached to the ventromedial wall. Rotation is probably complete.

C I. Vesicle well developed and oval in form. The dorsal wall is thick. The ganglion is attached to the lateral wall. No rotation.

C II. This vesicle from some cause is not closed and opens laterally. The opening doubtless represents the lateral wall. Wall is thickest ventromedially. Rotation is complete.

Examination 34 hours after operation.

A I (fig 15). The thinnest part of the wall turns toward the brain, the thickest ventromedially. Rotation is complete.

A II. Vesicle large and oval. Ventromedial wall thick. Rotation complete.

A III. Vesicle oval. The ventral wall and the lower part of the medial wall are thick. General appearance distinctly suggests rotation.

B I. Vesicle is too small for study.

B II. Vesicle well developed. Position difficult to determine.

B III. The vesicle is round. Ventromedial wall thick. Ganglion attached to upper part of medial wall. Rotation is complete.

C I (fig. 16). Vesicle slightly oval. The ventromedial wall is thick. Rotation complete.

C II. Proper criteria lacking.

ROTATION OF THE EAR VESICLE WHEN TRANSPLANTED TO A SPECIMEN OF A DIFFERENT ORDER

As already stated, the rotation of the ear vesicle has heretofore been studied only in the same individual or in individuals of the same species. No one has as yet touched upon the question as to whether vesicles, when transplanted in an inverted position to individuals of a different species or of a different order reveal the faculty of rotation. In other words, whether environmental influence may extend to the organ of another species. For studying this problem, *Rana* and *Amblystoma* were chosen, the former belonging to *Anura*, the latter to *Urodela*.

Transplanting vesicles from Amblystoma to Rana

The ear vesicles of *Amblystoma* were first transplanted in an inverted position to *Rana* and examined after two weeks. For judging the position, the endolymphatic appendage proved the most efficient criterion because of its special location between the brain and the labyrinth, and also because of its structure, consisting as it does of cuboidal cells. The semicircular canals may be also used as a guide. The lagena also takes a characteristic position, projecting backward from the ventromedial part of the vestibule. It is composed of intensely staining columnar cells.

The results of this experiment show, first of all that the ear vesicle of *Amblystoma* is transplantable to *Rana*. As the specimens were killed two weeks after the operation, it cannot be stated with certainty whether or not the vesicles would have developed to their final stage without any degeneration; but as far as can be seen from a histological study of this stage, no sign of degeneration could be noticed and the vesicles appeared quite normal. It is therefore probable that they would have continued to develop further. Coming back to the original problem, out of fourteen cases, eleven showed complete rotation; one case did not permit a decision, owing to its insufficient development; the other two cases contained no vesicles, the organs having probably slipped out. Thus complete rotation occurred in almost all of the specimens studied. The environmental influence is effective, therefore, even for the ear vesicles of species of a different order.

Upon microscopical examination, the normal vesicles of the two species give different impressions as to their characteristics of form, at least in the specimens examined two weeks after operation; but the transplanted vesicles seem to develop just the same as those of the host and not to retain the exact characteristics of those of the original animal. The same is true of the cellular constituents. For instance, there is an evident difference in the size of the cells in the two species, those of *Amblystoma* being larger than the cells of *Rana*.

Uhlenhuth ('13), in transplanting the eye from the larva of *Salamandra maculosa* to another larva in a younger stage of development, found that the transplant is not only delayed in its metamorphosis, but that the metamorphosis is exactly synchronous with that of the normal eye of the host, even to the smallest detail. It seems to me that in my heteroplastic transplantation the influence of the host upon the transplanted vesicle was more than synchrony. Since my experiment was planned for the study of inversion, it is not adequate for the solution of this question. Therefore I shall make but a brief reference to it and shall attempt to test its accuracy by further experiments and more detailed observations.

Transplanting vesicles from Rana to Amblystoma

Seven specimens were operated upon. In two development was abnormal; in two no vesicles were found upon later examination; in the remaining three cases complete rotation was established. The results of this experiment, therefore, like those of the previous one, fully justify the conclusion that environmental influences can act upon ear vesicles of a different species. The whole appearance and cellular constituents of the transplanted vesicles appear to be the same as those of the host.

Abnormal development seems to occur more frequently in this than in the former experiment. It cannot be asserted positively whether this is due to mere accident or to difficulty in transplantation from *Rana* to *Amblystoma*; that is, from a higher to a lower species.

Protocols of experiments

Transplantation from Amblystoma to Rana. Specimens A II, A III, B II, C I, C III, D II, and E I (figs. 17 to 20) show the labyrinths in normal position. The endolymphatic appendage lies between the brain and the labyrinth. Dorsal and anterior is the anterior semicircular canal; dorsal and posterior, the posterior semicircular canal; lateral, the lateral semicircular canal. From the lower and medial part of the labyrinth chamber the lagena buds out posteriorly. The acousticofacial ganglion is attached medially to the labyrinth.

B I. The endolymphatic appendage is in normal position. The lateral and posterior semicircular canals are normally formed, but there is no anterior semicircular canal. The labyrinth is a little abnormal, but is in normal position.

C II (fig. 21). The vestibule is exceedingly large. The endolymphatic appendage is in its normal position. The lateral semicircular canal is not sufficiently closed, being represented by a groove, but its position is normal. The anterior semicircular canal is not formed. The posterior semicircular canal deviates slightly from the normal position. The lagena is incompletely formed. The labyrinth is in normal position. The acousticofacial ganglion is attached in normal position.

D I. The anterior semicircular canal presents an appearance as if it had been pressed against the brain. The endolymphatic appendage is situated a little higher than normal. The lateral and posterior semicircular canals are in proper position. Below the labyrinth there is an enclosed cavity, the medial wall of which is covered by columnar cells. This cavity is separated from the labyrinth by a thin wall, and no connection between them could be observed. The nature of the cavity is not clear. Though abnormal in formation, the labyrinth is still in its normal position.

E II. The labyrinth vesicle has not developed far. The endolymphatic sac can be seen in normal position, but aside from this, the vesicle shows practically no differentiation. The wall is covered by a simple layer of flat cells, while the basal part is lined by several layers of flat cuboidal cells. Though the vesicle itself does not reveal the true characteristics of the developed ear, there is still sufficient evidence to justify the conclusion that it is in normal position.

D III. Differentiation of the semicircular canals has not as yet occurred. The vesicle is lined by a simple layer of epithelial cells which are flat in the upper part and take on a cuboidal form in the lower. Medioposterior to the vesicle is another which has no connection with it. The lower part is covered by a layer of columnar cells and the remaining part by cuboidal cells. The medial wall is disassociated. The nature of this second vesicle is not clear; it may or may not represent the developed stage of a surplus vesicle, such as was found in several cases in the study of inverted vesicles in the early stages.

A I, B III. No vesicle found.

Transplantation from Rana to Amblystoma. A I. Just at the medio-dorsal part of the anterior semicircular canal, and separated from it by a thin septum, there is a small vesicle composed of cuboidal cells, while the canal is lined, as usual, by a layer of flat cells. Judged by the nature of the cells and the location of this structure, it must represent a rudimentary endolymphatic sac. The other semicircular canals and the labyrinth are in normal position.

B II (fig. 22), C I. No endolymphatic appendages can be seen in their usual position. The semicircular canals are almost normal in form. Position normal.

A II. Vesicle shows no differentiation. It is too abnormal to determine its position.

A IV. The structure is too abnormal to admit of any definite conclusion.

B I, C II. No vesicle could be found.

INVERSION OF THE EAR VESICLE ON ITS TRANSVERSE AXIS

As already mentioned, Spemann in his experiments on inversion obtained a different result from those of Streeter. He inverted the ear vesicles of *Rana esculenta* about their transverse axes—that is, upside down—and after the operation kept a cover-glass on the wound for half to three-quarters of an hour to prevent the vesicles from slipping back. Out of twelve specimens operated upon he found that the vesicle remained in its inverted position in nine cases. In two only a ‘Bruchteil der Anlage’ was rotated, and in one case restoration of the original position was observed.

Concerning this discrepancy from Streeter’s results, the latter gave the following explanation: “Spemann raised a relatively large square flap of skin and exposed a larger area of the deeper structures, perhaps thereby injuring the environment in a way that lessens the postural interaction between it and the ear vesicle. In the second place the use of the weight may retard the movement of the vesicle and prevent its rotation.”

This naturally raises the question, What would be the result if inversion on the transverse axis were carried out by the method used by Streeter? To throw light upon this problem has been the purpose of the present study.

I operated upon seventeen animals in the manner already described, keeping the wound free from any weight after the opera-

tion. The specimens were fixed after ten days. The results were as follows: Rotation, 5; no rotation, 4; lack of criteria, 4; no vesicle, 1.

It is thus demonstrated that the ear vesicles under these conditions do not always rotate back to their normal position, even with the same technique used in cases which resulted in rotation. One can hardly assume that this discrepancy is due merely to a difference in the inversion axis, and the explanation must be sought in some other cause.

On account of the fact that the operation necessitates detaching the skin from the ear vesicle, the latter is usually broken open at its area of closure. If the vesicle is replaced inside out in its pocket, the smooth, intact surface of the original medial side comes in contact with the wound. The skin then grows gradually from all sides of the wound over the vesicle and finally covers it entirely, without any fusion between the two. The matter is different when the vesicle is replaced upside down. Here the wound of the skin and that of the vesicle come directly in contact. During the process of healing these two wounds may easily fuse with each other. Among Spemann's figures representing the ear vesicles in different stages, there is one which illustrates a vesicle which had just closed, and shows clearly the fusion of skin and vesicle. In explaining this figure Spemann states: "Die Epidermis zieht zweischichtig über das Bläschen hinweg, mit Ausnahme einer nicht ganz regelmässigen Stelle, wo vielleicht noch ein Zusammenhang zwischen beiden besteht."

Although a positive decision can only be obtained by actual study for a number of specimens in early stages, Spemann's figure suggests that this explanation may be the correct one.

My results and Spemann's differ as to the percentage of rotated vesicles, Spemann's experiments giving a majority of non-rotation. This difference can also be explained by fusion, which is apparently much more favored by Spemann's technique of keeping the wound free.

*Protocols of experiments**Transverse inversion.*

B II (fig. 23), F II. The endolymphatic appendage is situated normally between the vestibule and the brain. The semicircular canals are not well differentiated. The macula is located ventromedially. Rotation is complete.

D II. Differentiation of the semicircular canals is insufficient. The endolymphatic appendage is situated slightly posterior between the vestibule and the brain. Rotation is complete.

G II. The lateral and anterior semicircular canals are nearly in normal position. The posterior semicircular canal is not formed. The lagena is visible. The endolymphatic appendage is in normal position. Rotation complete.

G I. The endolymphatic appendage is in normal position. No differentiation of the semicircular canals has taken place. Rotation is complete.

C I. An oval sac arises from the lower part of the lateral wall of the vesicle and extends dorsally. The epithelium is not as flat as that of the vesicle, but at the blind end of the sac it takes on a cuboidal form. Communication with the main labyrinth takes place by a relatively large opening. Here the true duct is absent. These two vesicles are in close contact with each other. The whole structure is strongly suggestive of an endolymphatic sac. The semicircular canals are defective. By the position of the endolymphatic sac it can be said that the labyrinth remained in its inverted position.

C II (fig. 24). A pear-shaped endolymphatic sac buds out from the lower part of the lateral wall of the labyrinth and projects upward. Its cavity is incompletely divided by several septa and its wall is covered by cuboidal cells. A semicircular canal is found dorsally. Judged by the position of the endolymphatic appendage, this labyrinth must be still in its inverted position. The semicircular canal corresponds to the lateral semicircular canal.

E I. A vesicle is closely attached to the lateral side of the labyrinth, and communicates with the latter by a small aperture. The wall is composed of flat cuboidal cells. From its structure this vesicle doubtless represents an endolymphatic sac and not a semicircular canal. One semicircular canal is formed dorsally, and this is probably the lateral one. The labyrinth, therefore, has remained in its inverted position.

D I. There is no evidence of an endolymphatic appendage. In the medial side of the labyrinth the epithelium shows thickening. A semicircular canal is formed in the upper part of the labyrinth. As no endolymphatic appendage is visible, it is somewhat difficult to determine the position exactly. Generally, however, the macula occupies the ventromedial part of the labyrinth, whereas here it occupies the medial wall alone. This vesicle may therefore be said to have remained in its transplanted position; hence the dorsally situated canal must be in reality the lateral semicircular canal.

A I, A II, B I, F I. No endolymphatic appendage or semicircular canals are formed in any of these specimens. Accordingly, a decision as to the posture of the vesicles is difficult to reach.

E II. No vesicle.

DISCUSSION

In explanation of the environmental influences which cause the inverted ear vesicles to rotate back to their normal position, Streeter suggests three possibilities which may act either separately or in combination in bringing about this phenomenon.

First, the shape of the vesicle and the pocket into which it fits: "The auditory pocket is bounded by the different structures, which present different degrees of resisting pressure and act upon the ear vesicle from various directions. When the ear vesicle is abnormally placed the pressure compels the vesicle to turn back and keep the normal equilibrium. An objection to this explanation is the fact that vesicles having abnormal form and that could not possibly fit well in the usual pocket, right themselves almost as well as the normally shaped ones." This objection is hardly justified, since the vesicle might have turned back before it differentiated, as shown by findings in experiments upon early stages. The inverted ear vesicles in the early stages often are not of the regular oval form. Notwithstanding the fact that this irregularity would serve to prevent the vesicle from fitting into the original pocket, there were still many cases in which such vesicles did regain their normal position. Thus, according to my results, mechanical force alone cannot be regarded as the cause of rotation.

The second hypothesis advanced by Streeter is that the nerve fibers which connect the ganglion mass with the brain may serve to draw the vesicle into its proper position. My studies have demonstrated that the ear vesicle can complete its rotation at a stage when the nerves have not yet grown out from the ganglion, which is closely attached to the vesicle; accordingly, there is no direct connection between the brain and the vesicle. This, therefore, cannot be considered as the sole cause of rotation.

The third possibility is mutual influence between the vesicle and the environment, direct or indirect, in a chemotactic sense.

Different parts of the vesicle and its environment may act upon each other differently. A certain part of the vesicle might have a special or stronger positive or negative affinity for a certain structure of the environment. By this force the vesicle may adjust itself to its normal posture. Since, however, the true nature of chemotaxis is still unknown, we should hesitate to accept it as a radical explanation. There arises now a question as to whether the environmental influence is specific for the place where the ear vesicle is normally present; in other words, whether the inverted vesicle can regain its normal position in regard to the axes of the body when transplanted in an abnormal place. I carried on some experiments along these lines transplanting the ear vesicles in an inverted position under the skin near the tail bud. Left in this position two weeks they gave no indication of differentiation, but presented dropsical vesicles. I then examined vesicles operated on in this way two days after operation. At this time they showed no degeneration and a difference in the thickness of the wall was discernible. I shall not, however, discuss this problem until I have made further experiments.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

Fig. 1 15 to 30 minutes after operation. B, vesicle open medially. No rotation. $\times 100$.

Fig. 2 $5\frac{1}{2}$ hours after operation. E, vesicle open ventrally. Half rotation. $\times 100$.

Fig. 3 16 hours after operation. B I, no rotation. $\times 100$.

Fig. 4 16 hours after operation. D II, rotated. $\times 100$.

Fig. 5 20 hours after operation. A II, rotated. $\times 100$.

Fig. 6 22 hours after operation. A I, no rotation. $\times 100$.

Fig. 7 24 hours after operation. D III, rotated. $\times 100$.

Fig. 8 24 hours after operation. E I, rotated. $\times 100$.

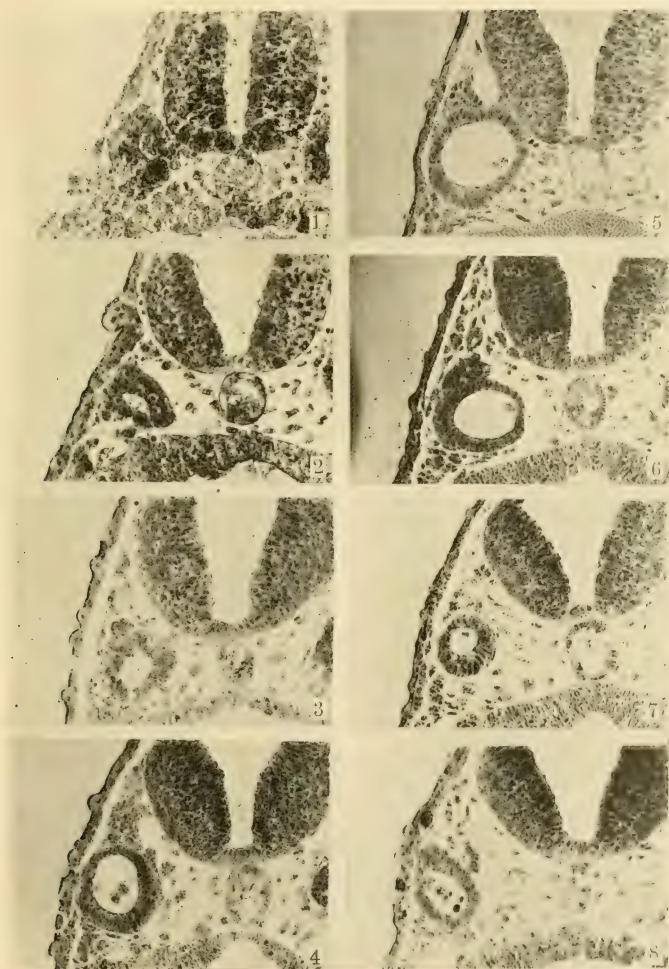


PLATE 2

EXPLANATION OF FIGURES

- Fig. 9 24 hours after operation. E II, rotated. $\times 100$.
Fig. 10 26 hours after operation. D I, rotated. $\times 100$.
Fig. 11 26 hours after operation. D II, no rotation. $\times 100$.
Fig. 12 28 hours after operation. A III, rotated $\times 100$.
Fig. 13 28 hours after operation. B III, rotated. $\times 100$.
Fig. 14 30 hours after operation. A II, no rotation. $\times 100$.
Fig. 15 34 hours after operation. A I, rotated. $\times 100$.
Fig. 16 34 hours after operation. C I, rotated. $\times 100$.

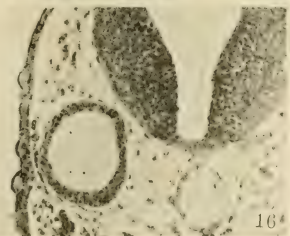
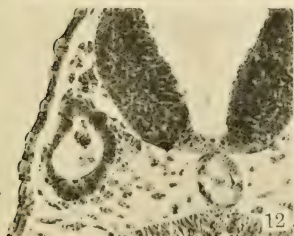
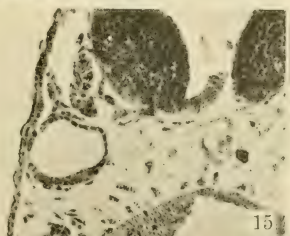
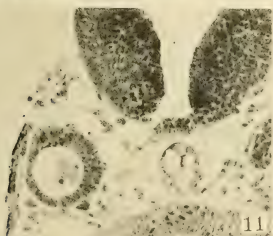
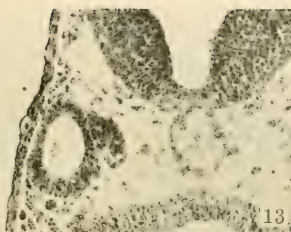
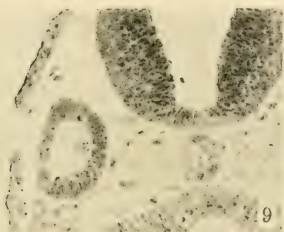


PLATE 3

EXPLANATION OF FIGURES

Fig. 17 Ear vesicle of *Amblystoma* transplanted to *Rana* in reversed position. A III, two weeks after operation. Normal position regained. Lateral and anterior semicircular canals visible. Between the anterior semicircular canal and the brain a small vesicle can be seen which, from its form and structure, is judged to be the endolymphatic appendage. The acousticofacial ganglion is attached medially to the vestibule. $\times 66$.

Fig. 18 Ear vesicles of *Amblystoma* transplanted to *Rana* in reversed position. A II, two weeks after operation. Normal position regained. Endolymphatic appendage and lateral semicircular canals visible, the former located between the crus commune and the brain. The ganglion also in normal position. $\times 66$.

Fig. 19 A section from a different part of the same specimen. Normal position regained. Posterior and lateral semicircular canals and lagena visible. $\times 66$.

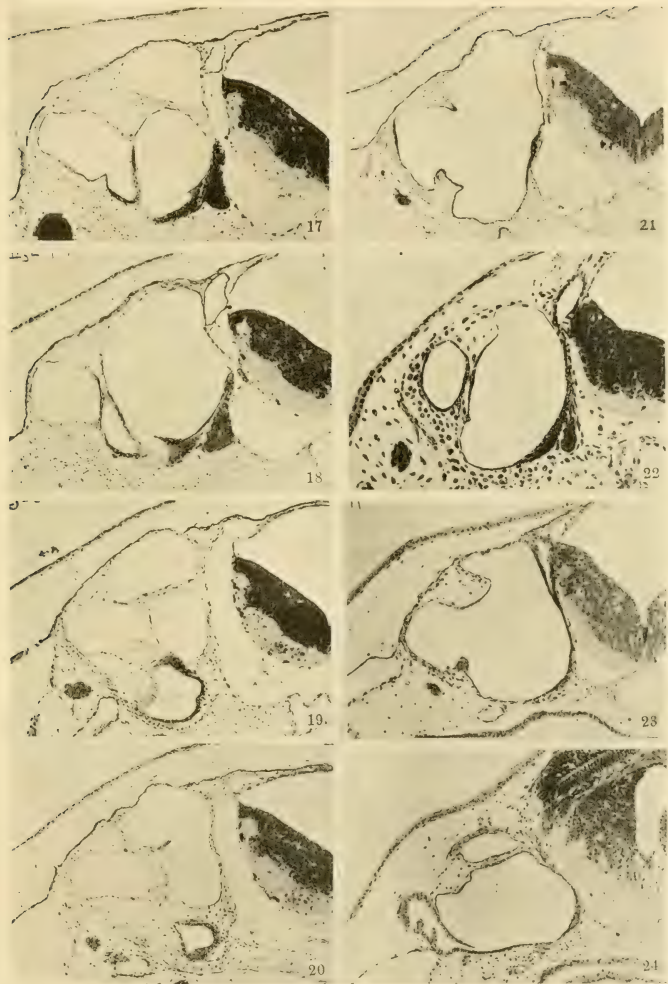
Fig. 20 Ear vesicle of *Amblystoma* transplanted to *Rana* in reversed position. C I, two weeks after operation. Normal position regained. Posterior and lateral semicircular canals and lagena visible. $\times 66$.

Fig. 21 Ear vesicle of *Amblystoma* transplanted to *Rana* in reversed position. C II, two weeks after operation. Normal position regained. Abnormal development. Endolymphatic appendage visible. $\times 66$.

Fig. 22 Ear vesicle of *Rana* transplanted to *Amblystoma*; B II. Normal position regained. Development practically normal. $\times 66$.

Fig. 23 Ear vesicle of *Rana* 10 days after operation. Inversion on the transverse axis. Rotated. $\times 66$.

Fig. 24 Ear vesicle of *Rana* 10 days after operation. Inversion on transverse axis. No rotation. Endolymphatic appendage lateral; lateral semicircular canal dorsal. $\times 66$.



Resumen por el autor, Edward C. Day.

La fisiología del sistema nervioso de los tunicados.

II. La relación del nervioso ganglionar con el corazón.

La actividad normal del corazón del tunicado *Ascidia mentula* exhibe series alternadas de contracciones designadas como de dirección ventral y dorsal, y las 25 contracciones de una serie requieren 2 minutos y 5 segundos. La rapidez inicial de la contracción es de 5.5 segundos por contracción o sea, 55 segundos para las diez primeras pulsaciones de una serie. Esta rapidez inicial y el número de contracciones fueron estudiados con referencia al efecto que pueda causar en ellas la estimulación o extirpación del sistema nervioso. La separación de pedacitos de la túnica, la sección de los nervios situados entre el ganglio y el corazón y la extirpación del ganglio supone un aumento de las contracciones desde 25 a 50 o 90, y la rapidez disminuye desde 5.5 a 5 o 5.4 segundos por contracción. La rapidez de la contracción se acelera de este modo, aumentando el número de contracciones y como resultado, dilatando la duración de una serie. Los efectos de las estimulaciones y operaciones desaparecen al cabo de una hora o un día, y el corazón vuelve a su condición normal originaria, manifestada por la rapidez de la contracción y número normal de pulsaciones. De los datos obtenidos no puede deducirse si el corazón está bajo la influencia del ganglio nervioso.

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THE PHYSIOLOGY OF THE NERVOUS SYSTEM OF THE TUNICATE

II. THE RELATION OF THE NERVE GANGLION TO THE HEART

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INTRODUCTION

In a previous paper (Day '19) the author showed that while the ganglion of the tunicate exerts certain coördinating and tonic effects upon its sensory reactions, they are not wholly under the control of the ganglion. Since it is also an established fact that the heart of the tunicate is capable of beating not only independently of the nerve ganglion, but apart from and independently of the body as well, the question arises as to whether the ganglion dispatches accelerator or inhibitory impulses to the heart, and whether it in any way augments or diminishes the vigor of the rhythmical contractions, or whether the heart is entirely unregulated by any ganglionic influence.

It was hoped that a careful recording of the beat under varied experimental conditions with the aid of a kymograph would reveal whatever influence the ganglion might exert.

MATERIALS AND METHOD

Ascidia mentula was the tunicate employed throughout the present investigation.

The body of *Ascidia mentula* is transparent enough as a rule to permit the heart to be seen when the animal is placed on its side in a vessel of sea-water and viewed through a binocular microscope. In order to observe it more readily, however, a slice was shaved off the tunic after a record had been made of the normal beat. This operation itself temporarily affected the rate, and a day was allowed to elapse for the heart to regain the normal before observations were continued. New records were then made of the normal condition, following which the heart was cut off from all communication with the ganglion either by severing the nerves or by extirpating the ganglion.

The pulsation-waves were recorded on a kymograph by ticking a Morse key every time a pulsation wave traveled beneath an arbitrarily chosen landmark on the sliced area of the tunic. With the aid of a metronome a time-curve in seconds was recorded directly beneath the heart-curve.

The temperature of the water in the vessel in which the tunicate lay fluctuated a degree or two with changes in the room temperature, but these slight variations had no measurable effect on the beat of the heart.

The records for a given animal extended over a period of from five to ten days. During this period the tunicates were kept in large aquaria provided with running sea-water. Whereas a mild form of stimulation, such as tactile, might be suspected of affecting the heart reflexly, attention was directed to extreme methods of stimulation first to determine whether they had any effect on the character of the beat. After first considering the features of the normal beat, the results of strong stimulation by cutting into the tunic, severing nerves, excising the ganglion and amputating a siphon will be presented.

NORMAL BEAT OF THE HEART

The heart-beat of an *Ascidia mentula* lying undisturbed in a vessel of fresh sea-water exhibits the following characteristics:

1. Alternating ventrad and dorsad series of pulsation-waves.
2. Each series consists of about the same number of waves, twenty to forty according to the size and condition of the animal.
3. Each requires about the same length of time for its completion, two to four minutes depending on the length of the series.
4. Each pulsation-series is followed by a pause of from ten to fifteen seconds before reversal occurs.
5. An acceleration of beat occurs usually during the course of a series.

The heart is looped around the base of the pharyngeal sac in the posterior end of the body in such a way that one end lies under the nerve ganglion and dorsal and the other end lies on the opposite side of the pharyngeal sac and ventral. A ventrad series, therefore, is one in which the pulsation-waves originate at the dorsal end and travel around the loop to the ventral end, and a dorsad series is one in which the direction of the waves is reversed, commencing at the ventral end and traveling around the loop to the dorsal.

The graphic records have been analyzed and the results set forth in the accompanying tables. Graph 15¹ of table 1 represents a typical sequence of events for the heart-beat of a normal, uninjured, and unstimulated animal. The first line states the number of beats in alternating ventrad and dorsad series, and the next three lines give an analysis of the first ventrad and of the first dorsad series of beats.

The ventrad series required 2 minutes and 19 seconds for the 25 beats comprising it. This interval of time was distributed over the series as follows: 55" for the first 10 beats, 55" for the second 10 beats, and 29" for the last 5 beats of the series. A pause of 18" followed, and succeeding this came a normal reversal with the beats traveling dorsad. The dorsad series required 54" for the first 10 beats, 55" for the second 10, and 28"

for the last 5, totaling 2 minutes and 17 seconds. After a pause of 8", a reversal again occurred.

It will be noticed that there is close correspondence between the time-relations for the two series except in the matter of the intervening pauses. The fact that the landmark selected on the tunic as the point for observing the passage of the impulses, did not lie equidistant from the two ends of the heart was found, however, to account for this difference in length between the pause following a ventrad series and the pause following a dorsad series.

The beat of the heart varied from day to day even under normal conditions as may be seen from a glance at table 4. For *Ascidia mentula* no. 15 the number of beats per ventrad series ranges from a minimum number of 25 up to a maximum of 42, and the number per dorsad series ranges from 21 to 35. These extremes in the range of beats are no doubt the expression of varying degrees of stimulation of some sort to which the animal is subjected. The average number of beats per series, ventrad and dorsad alike, when the extremes are disregarded is 25. There are, however, individual differences, as comparison of the records of the three animals in the table reveals. The normal number of beats runs higher for both nos. 16 and 18, the latter having the highest average record of the three.

The fifth feature listed for the normal heart-beat, acceleration during the course of a series, is best seen in a long series such as is exhibited by *Ascidia m.* no. 18. In graph 18¹, table 3, the first ventrad series has 39 beats which required 3 minutes and 23 seconds for their execution. The number of seconds per every consecutive 10 beats runs 53, 51, 49, and then 50" for the last nine beats. The second and third 10 beats, therefore, required less time than either the first 10 or the last 10 of the series. In the case of the first dorsad series where the 38 beats required 3 minutes and 30 seconds, the acceleration is less pronounced, the third 10 beats requiring but one second less than the first 10 and seven less than the last 10. In both instances it takes the first 30 beats in which to develop the acceleration and the last 8 or 10 beats to check it. In other words, there is a rather

abrupt retardation of beat at the close of the series. If plotted as a curve the result would take the form of a skew curve with a gradual ascent and an abrupt descent.

In graph 18⁷, in which the ventrad series has been lengthened as a result of injury, the series of figures 45, 46, 44, 44, 44, 45, 45, 46, (60) would yield a more nearly symmetrical curve, showing that the acceleration had had time to develop to a maximum and decline before its development was cut short by a reversal.

OPERATIONS AND THEIR EFFECT ON THE BEAT

The operations performed on the animals consisted in slicing away the tunic over the region of the heart, making incisions for the purpose of severing the visceral nerve strand, and extirpating the ganglion. All of these procedures result in a lengthening of the pulsation series by increasing the number of beats. Furthermore, the rate of beat is increased about 20 per cent; ventrad and dorsad series follow each other at shorter intervening intervals; and the acceleration which was only mildly expressed in the graphs for the normal beat, crops out strongly as a result of the vigorous stimulations from the operations.

Graph 18⁷ exhibits all four of these phenomena as a result of slicing away a part of the tunic. The animal previously (three days before) had the ganglion removed, and consequently had not entirely recovered, but the effect of the slicing is apparent in the ventrad series where the series has been lengthened from 68 to 81 beats, the initial rate has dropped from 51 to 45 seconds for the first 10 beats and the rate in the middle has become 10 per cent faster than it is at either end. This happens to be the only graphic record made for the effects of slicing the tunic. The operation was usually performed at the beginning of the investigation in order to render the heart more visible for making the records. From unrecorded observations, however, I can confirm the results of this single graphic record.

The result of severing nerves is seen in graphs 15³ and 18². If the ventrad series of these is compared with the ventrad series of those of the preceding normal graphs, 15¹, 15², and 18¹, the

effects are readily seen to be the four already mentioned: in 15³ the number of beats jumps from 30 to 57, in 18² from 30 to 54; in 15³ the initial rate drops from 58 to 42, in 18² from 53 to 51, and in both instances the acceleration crops out and tends to reach a maximum nearer the middle of the series than was possible in the shorter normal series. The dorsad series of 15³ exhibits, as a result of severing the nerves, a very marked increase in the number of beats from 21 in the preceding normal series to 126, and also a decided speeding up in the rate of beat from 61 to 44 seconds for the initial 10 beats. The maximum rate is attained early in the series at the end of the third 10 beats. In 18², whereas there is little change in rate, there is an increase in length of the series from 38 to 54 beats.

Extirpation of the ganglion produces effects similar to those wrought by paring the tunic and severing nerves. They are quickly apparent from a study of graphs 15⁶, 16⁴, and 18⁵. The number of beats for the ventrad series increases in the three graphs from 25 to 83, 27 to 85, and 42 to 65, respectively; for the dorsad series from 23 to 54, 25 to 95, and from 41 to 58. The increase in acceleration, as indicated by the difference in the number of seconds required for the initial 10 beats, for the ventrad series is 14", 13", and 4", respectively, and for the dorsad series 11", 9", and 8" for the three graphs under consideration. The duration of the three ventrad series lengthens from 2' 29" to 6' 21", 2' 27" to 5', and from 3' 52" to 5' 10", the three dorsad series from 2' 11" to 3' 52", 2' 19" to 6', and from 3' 39" to 4' 41". There is further a shortening of the pause between the ventrad and dorsad series. Owing to irregularities attendant upon the choice of a landmark for observing the pulsations, the average of two successive pauses is selected for comparison rather than single pauses. In graphs 15⁶ and 18⁵ the averages are 8" and 10.5", respectively. No average could be obtained for graph 16⁴ owing to interruption of the observations before the dorsad series was completed. The single pause recorded, however, is 3" less than that for the previous normal series. Thus, not only do the individual beats follow each other in more rapid sequence, but each series of beats succeeds the one preceding at a shorter

intervening interval. In the dorsad series of both 16⁴ and 18⁵ and in both ventrad and dorsad series of 15⁶ there is a retardation of rate after the initial acceleration with a subsequent speeding up before the final slowing down. This is exhibited best by graph 15⁶ in the ventrad series 43, 44, 48, 47, 47, 46, 48, (50) and in the dorsad series 43, 44, 43, 41, 42, (47), the places of retardation being indicated in italics. The figures in parentheses represent the calculated number of seconds for the incompleting last 10 beats and they indicate that the final rate was slower than at any other place in the series.

From these results it is evident that the three operative procedures of paring away the tunic, severing the nerves and extirpating the ganglion, bring about in lesser or greater degree the same alteration of the characteristics of the dorsad and ventrad series of beats: an increase in the number of beats, a primary and secondary phase of acceleration through the series, a shortening of the interserial pauses, and a lengthening of the time of the series more or less directly in proportion as the number of beats is increased and their frequency accelerated.

When, however, the more profound operation of amputating the oral siphon is carried out subsequent to the removal of the ganglion, as was the case for graph 18⁸, the above results occur in a considerably modified form. The accelerative effect is more strongly expressed here than in any of the preceding graphs, but the number of beats is incommensurately small. Mark, for example, how the rate for the initial 10 beats gradually increases with each succeeding injury: from the normal 53" in the ventrad series of 18¹ it drops to 51" in 18², to 50" in 18⁵, to 45" in 18⁷, and to 44" in 18⁸. Now, up to the third injury in 18⁷ there is a concomitant increase in the number of beats from the normal number 39 in 18¹ to 54 in 18², to 65 in 18⁵, to 81 in 18⁷ but in 18⁸ they drop back to 65. One would have expected, from the way the acceleration of rate continued to increase, that the number of beats would here have jumped from 81 to about 85, but instead it drops back to 65 a figure corresponding to an initial rate of acceleration per first 10 beats exhibited by 18⁵. In the dorsad series this drop is even more pronounced: the 22 beats

for the series is not only a striking contrast to the 78 beats of the preceding graph, but also to the 38 beats for the normal uninjured condition exhibited by the first graph of the set, 18²; the number, instead of progressing from 78 to about 85 or 90 *pari passu* with the increase in rate from 49 to 44 seconds for the initial 10 beats, has fallen way below the normal.

Since the amputation of the siphon entailed considerable loss of blood, this abrupt shortening of the series by decrease in number of beats must in some way be correlated with that loss. It is conceivable that the length of a pulsation series may be influenced by different factors, of which one is blood pressure, another the exhaustion of anabolic materials, a third the accumulation of the products of catabolism, and a fourth nerve reflexes, but to which one is to be attributed the diminished number of beats cannot at this point be taken up for discussion.

The fact to be emphasized, however, in connection with this particular operation is, that the rate of beat has gone on increasing according to expectation, whereas the number of beats and the length of the series has not.

RECOVERY FROM OPERATIONS

The day following the extirpation of the ganglion the animals exhibited a heart-beat which was quite normal in every respect. The pulsation-series had shortened, the rate of beat had slowed down, the intervening pauses had lengthened again, and the strong acceleration which had cropped out in the longer series as a result of injury had disappeared except for the same faint trace characteristic of the normal beat.

A comparison of the recovered condition in graph 15⁷, table 1, with the initial normal condition in graph 15¹ reveals precisely the same number of beats in the ventrad series of both, namely 25, in contrast to 83 beats for the state of injury resulting from removal of the nerve ganglion; and in the dorsad series 28 and 23 beats for recovery and normal in contrast to 54 beats for the state of injury. For the recovered condition the rate of the ventrad series runs 51, 51, 27 as compared to 55, 55, 29 for the normal condition, and both the recovered and the normal condi-

tions are in decided contrast to the rate of 43, 43, 44, 48, 47, 47, 46, 48, 15 for the injured condition. The recovered rate of the dorsad series is 51, 54, 40 as compared to 54, 55, 28 for the normal, and here again the rate for the recovered and normal conditions are in sharp contrast to the rate of 43, 44, 43, 41, 42, 19 immediately after the ganglion had been removed.

While in graph 15⁷ the recovered rate did not slow down quite to the normal in either ventrad or dorsad series, in graph 16⁵ the rate of both series after recovery was a trifle slower than normal. Here the ventrad series shows after recovery from the operation the same number of beats, 21, as for the normal condition exhibited by graph 16², but these 21 beats required 6 seconds longer for their completion in the recovered condition than they did in the normal series. The dorsad series is also subnormally slow. Since no record was made upon the day following the extirpation of the ganglion for animal no. 18 no reliable statement can be made regarding the character of immediate recovery. Three days after the operation, however, a graphic record, 18⁶, revealed a more advanced degree of injury, as far as number of beats is concerned, than did the record made immediately following the operation. But whereas the number of beats had increased from 65 (injured ventrad series) to 68 (recovered ventrad series) and from 58 (injured dorsad series) to 83 (recovered dorsad series), there was a slight recovery to a slower rate of beat for both ventrad and dorsad series, as the graphs 18⁵ and 18⁶ of table 3 show.

SUMMARY OF RESULTS

There is a certain normal activity of the heart of the tunicate, *Ascidia mentula*, in which the number of beats is about 25 for both ventrad and dorsad series. The initial rate is 5.5 seconds per beat, and the pauses between the series average about 13 seconds. Stimulation, such as slicing pieces off the tunic, severing the nerves between ganglion and heart, and extirpating the ganglion, causes the number of beats to increase from 25 to 50 or 90, the rate to drop from 5.5 to 5 or 4.5 seconds per beat, indicating acceleration, and the pauses to shorten from 13 to 10 or 8 seconds. In addition to these changes there is a retarda-

tion of the beat which manifests itself near the middle of a series. These changes go hand in hand with one another, and when any one of them is patently present, the others are present too. Exceptions occur in cases where the circulation has been interfered with through operation on the ganglion.

The effects of stimulation abate and disappear in the course of an hour or a day, depending on the character of the operation, and the beat of the heart again exhibits its original normal characteristics.

DISCUSSION OF DATA

It is important to remember before entering upon a discussion of the foregoing results that there was at least one feature of the heart-beat which the method adopted for experimentation did not permit an analysis of, namely, the vigor of the contractions.

The features which were capable of being analyzed, however, were the number of beats per series, the frequency of beat, and the length of the interval between alternating series. Since the problem was to ascertain what control the ganglion exerted over the heart, each of these features of the beat may now be considered for the evidence that it contributes toward the solution of that problem.

One must first determine which of these features is the most delicate indicator of the changes in beat due to stimulation. Even though the features mentioned are closely correlated as expressions of changes in heart activity, it may well be inquired whether they are all of equal value or not as indicators of the quantitative aspect of these changes. The feature of the length of the interserial pauses is of minor consequence in comparison with the other two features and will be omitted from the discussion. The question therefore resolves itself into one of whether the alteration of the rate of beat or alteration of the number of beats in a series is the best indicator of the functional state of the heart, for to the most delicate indicator one must turn for the nicest evidence of any ganglionic control that may exist.

A comparison of graphs 18² and 18³ furnishes an answer to this question.

The dorsad series of 18² shows a condition almost the reverse of that for the ventrad series of 18⁸. In the latter graph the rate of beat has increased and the number of beats has been reduced, whereas in the former graph the number of beats has been increased while the rate of beat has been reduced. Now, as a rule, the number and the rate as both increased by stimulation, but here we have the two features changing not only independently of each other, but in opposite directions. Apparently it is due to a greater volatility or evanescence of the one phenomenon in comparison with the other: the change of rate is the more evanescent and volatile while the change in the number of beats possesses greater inertia; consequently, the rate waxes and wanes while the number of beats continues to increase, and this change crops out in a graph either as an acceleration or a retardation during the time that the number of beats is developing to a maximum.

The rate of beat would seem, therefore, to be the more susceptible of the two phenomena to stimulation and might be regarded as the best indicator of the state of excitation. One would consequently expect to find that if there were any regulatory control exerted by the ganglion, it would be first expressed by this delicate indicator. The change in rate of beat may be regarded as the 'vernier' for determining the finer measure of any existing control, while the number of beats may be regarded as the coarser scale for measuring its grosser aspects.

The number of beats, as has been pointed out, increases as a direct effect of stimulation, but, irrespective of whether the stimulus has been the result of incisions in the tunic or of complete removal of the ganglion, the number subsides again to normal after sufficient time has elapsed for recovery. If, after recovery, the animal is again stimulated, the same rise in number of beats occurs with a subsequent reversion to normal after the stimulation effects have died away. Animals nos. 15 and 16 show this recovery the best. In graphs 15⁴ and 15⁷, both of which are cases of recovery, the first after slicing the tunic and the second after removing the ganglion, the number of ventrad beats has dropped from 57 and 83, respectively (graphs 15³ and 15⁶),

back to the normal 25 of graph 15¹. In graph 16⁵ is also seen the restoration to normal, 21 beats, after the high increase to 85 beats exhibited by the preceding graph as a result of removing the ganglion. The dorsad series shows a normal of 25 in graph 15¹ and a recovery to 24 and 28 beats, respectively, in graphs 15⁴ and 15⁷. The dorsad series of 16³ *b* also shows a normal of 25 beats, but after recovery from the effects of the ganglion the number of beats falls short of the normal 21 and 22 beats in 16⁵ *a* and 16⁵ *b*, respectively, and 19 beats in 16⁶. Thus, except for the 28 beats in graph 15⁷, the 24, 21, 22, and 19 beats are less than the normal 25 which was selected as the shortest normal dorsad series for comparison.

These instances substantiate the statement that the number of beats returns to normal or approximately to normal the day following extirpation of the ganglion. The ventrad series approximates the normal more closely than the dorsad series does in this respect.

It must here be remarked that the normal number of beats is itself by no means constant: the number ranges for animal no. 15 from 25 to 30 for the ventrad series and from 21 to 25 for the dorsad; for animal no. 16 it ranges from 21 to 38 for the ventrad and from 25 to 33 for the dorsad. These fluctuations of the normal number of beats may be attributed to minor stimulations of some sort or to variations in the general metabolism. It is to be expected, therefore, that similar fluctuations would occur after recovery.

Now, in the cases above mentioned where the recovered condition was 19, 21, 22, and 24 beats instead of the normal number 25, could this shortage be interpreted as a result of the removal of the ganglion and with it a removal of a tonic influence which, under normal conditions, enables the heart to relax to a state of moderate activity?

The answer to this question hinges upon whether 25 in the graphs for animal no 16 is the lowest number of beats possible for a normal dorsad series. While it is the lowest on record for this animal, it is not the lowest for animal no. 15, where the normal is as low as 21 beats to a series. This is the case for

graph 15°. After severing the nerves, however, the number of beats after soaring to 126 returned to 23, and when the whole ganglion was removed it rose to 54 and subsided to 28. In this instance the recovered condition exhibits a number of beats which is above the normal and it stands in contrast to the recovered condition for animal no. 15, where the number of beats was below the normal.

One may therefore just as legitimately ask whether this supernormal number of beats after recovery is likewise not an expression of lifted ganglionic control.

In the absence of conclusive evidence from the study of the dorsad series, one can only balance the two cases one against the other, pointing out that animal no. 15 recovers at best to a number of beats only two above the lowest recorded normal, while animal no. 16 recovers at best to a number six below the lowest recorded normal. But in view of the fact that the normal number fluctuates through a recorded range of 8 beats for no. 16 and 4 beats for no. 15, the two cases of recovery may be regarded as close enough to the normal to be within the range of normal fluctuation. This is substantially confirmed by the instances pointed out in the records of the ventrad series where the number of beats upon recovery coincides exactly with the normal number. Animal no. 15 shows this precise readjustment both after severance of nerves between ganglion and heart and after extirpation of the ganglion: three times the heart upon recovery exhibits the same number of 25 beats which it exhibited prior to the operations. Animal no. 16 twice shows a state of recovery in which the number of beats tallies with the normal record of 21.

Consequently, since there is not only the close approximation to the normal number of beats upon recovery in the dorsad series, and also an exact tally with the normal upon recovery in the ventrad series, the feature of the number of beats gives no evidence of any regulatory control exerted by the ganglion on the heart.

The manifestation of fluctuations in the number of beats about a certain normal both for the uninjured and the injured state of the animal suggested the possibility that the range of

fluctuation might be different for the two conditions. Table 4 was consequently prepared to test this possibility out: consecutive ventrad series are brought together in one column and consecutive dorsad series in another and the ranges of fluctuation placed beside them so that the extremes may be picked out at a glance.

The least fluctuation exhibited by normal animals are those in graph 15¹, where the variation in three consecutive series of both ventrad and dorsad beats is a single beat, and in the dorsad series of 16¹, where it is also a single beat for two consecutive series. But the same fluctuation in consecutive series occurs likewise immediately after the nerves between ganglion and heart have been severed, as in the ventrad set of 15², and after recovery from extirpation of the ganglion had occurred, as in the dorsad series of 16⁶; in both cases the fluctuation between two consecutive series is but a single beat.

The maximum fluctuations that occur in normal consecutive series of ventrad beats are 12 for animal no. 15, 17 for animal no. 16, and 2 for animal no. 18; and in normal consecutive dorsad series they are 14, 3, and 3 beats for the same animals, respectively. The maximum is much higher after some of the performed operations: 24 beats in the dorsad set of 15 where the nerves had been severed, 26 in the dorsad set of 18 after the same operation, and 30 beats in the ventrad set of 18 where the ganglion had been removed. In several instances no data were obtained for consecutive series following operations, owing to the interruption of the observations.

The fluctuations in consecutive series after recovery had taken place range from the minimum of one beat to 2, 3, 4, 5, and 9 beats as seen in graphs 18, 16, 15 and 18, respectively.

It is evident from the foregoing that fluctuations both small and large occur in sets of consecutive series whether ventrad or dorsad, irrespective of whether the animal was in the normal condition or recovered condition or in a state of injury. The fact that greater fluctuations occur in the normal than the recovered condition might be interpreted as indicating a greater sensitivity, the source of which could be attributed to the pres-

ence of ganglionic influence. Final judgment, however, must be suspended until the remainder of the evidence has been considered.

In table 5 has been brought together the rates in seconds per every 10 beats. The first or upper portion of the table gives the rates for ventrad series and the last or lower portion the rates for dorsad series. For animal 15 the normal rate was 55", 55", (29") for one reading, and 58", 55", 53" for a second ventrad series. The number in parenthesis is for the last incomplete set of beats, here five instead of ten. There is a fluctuation of 3 beats between the initial sets 55 and 58 for the two readings. Of the six readings for the normal beat of no. 16 the figures range from a minimum of 54 to a maximum of 69. Since the third reading was exceptionally high, the average initial rate would be 57" for the first ten beats. Animal no. 18 shows 53" for the single normal reading obtained.

When the nerves between ganglion and heart were severed, the initial figure dropped to 42 for no. 15, to 51 for no. 18, indicating by the fewer seconds required in each case for the initial 10 beats a more rapid rate of beat. No. 15 thus shows the greater acceleration of the two as a result of the operation.

The following day, however, after recovery had occurred, the rates had returned to 53" and 57" for no. 15 and to 57" and 54" for no. 18. In the first case 57" lies between the 55" and 58" of the normal reading above, and 54" in the second case is but one second greater than the normal.

When the ganglion was extirpated the initial figure again dropped to 43" for no. 15, 42" for no. 16, and to 50" for no. 18, indicating renewed acceleration. Recovery was recorded for the first two animals only: for no. 15" it returned to 51 and for no. 16 to 70". In the case of no. 15 the return was not quite to the normal, while for no. 16 it was to a figure higher than the average normal; i.e., in the first case the recovered rate was still slightly accelerated, while in the second it was slower than normal. If the last reading for the recovered rate of no. 16 be compared, however, to the third normal reading, they are nearly alike: 69, 67, (7) vs. 69, 64, (7). Since the dorsad series

closely resembles the ventrad series with respect to these effects of operations and recovery upon the initial rate and since the results are even closely akin numerically, it must be concluded that alterations of the initial rate give no evidence of any control exerted by the ganglion.

TABLES

Tables 1 to 3 were compiled from kymograph records. The records were obtained by ticking off the passage of the waves of contraction through the instrumentation of a Morse key in circuit with an electromagnetic signal.

For each graph thus secured there is given a statement in the table of the number of beats occurring in successive alternating ventrad and dorsad series, V standing for ventrad and D for dorsad; and then an analysis of the italicized pair of these, showing the time-rate for every ten beats of each series, together with the pause intervening between two consecutive series.

Table 4 is a rearrangement of the data contained in tables 1 to 3 for the sake of comparing, first, the number of beats in the ventrad series for all three animals and in the dorsad series for all three animals, and, second, the range in fluctuation for both ventrad and dorsad series of beats.

Table 5 is arranged to bring together the time-rates for comparison. The first six rows give the rates for the ventrad series of all three animals and the last five rows give the rates for the dorsad series.

TABLE I

Ascidia mentula, no. 15

Graph 15 ¹ . 5.V.13 Pulsation series: 25V-25D-26V-26D-26V-26D									
Normal.....	1st Ventrad series (25V)				1st Dorsad series (25D)				
Beats.....	10	10	5	= 25	Pause	10	10	5	= 25
Seconds.....	55	55	29	= 2'19"	18"	54	55	28	= 2'17"
Graph 15 ² . 8. V. 13 Pulsaton series: 21D-30V-30D-42V-35D									
Normal:.....	1st Ventrad series (30V)				1st Dorsad series (21D)				
Beats.....	10	10	10	= 30	Pause	10	10	1	= 21
Seconds.....	58	55	53	= 2'46"	15"	61	64	8	= 2'13"
Graph 15 ³ . S. V. 13.a.m. Pulsation series: 126D-57V.....58V-102D									
Injury 1*	1st Ventrad series (57V)				1st. Dorsal series. (126D.)				
Beats... 1010101010	7	= 57	Pause	10	10	10	10	10	1010101010 6 = 126
Seconds. 42 40 39 40 40 30	= 3'51"	10"	44	43	42	44	44	44 45 45 45 45 48 29	= 9'22" 8"
Graph 15 ⁴ . 8.V.13.p.m. Pulsation series: 23V-22D-25V-24D-28V-26D									
Recovery	2nd Ventrad series (25V)				2nd Dorsal series (24D)				
Beats.....	10	10	5	= 25	Pause	10	10	4	= 24
Seconds.....	53	53	25	= 2'11"	15"	52	52	23	= 2'7"
Graph 15 ⁵ . 9.V.13.p.m. Pulsation series: 20D-23V-23D-25V-25D-26V-25D									
	2nd Ventrad series (25V)				2nd Dorsal series (23D)				
Beats.....	10	10	5	= 25	Pause	10	10	3	= 23
Seconds.....	57	56	27	= 2'20"	18"	54	58	19	= 2'11"
Graph 15 ⁶ . 9.V.13.p.m. Pulsation series: 54D-83V-68D									
Injury 2*	1st Ventrad series (83V)				1st Dorsad series (54D)				
Beats... 10	10	10	10	10	10	10	10	10	4 = 54
Seconds... 43	43	44	48	47	47	46	48	15	= 6'21"
									8"
									43 44 43 41 42 19 = 3'52"
									8"
Graph 15 ⁷ . 10.V.13 Pulsation series: 21V-26D-25V-28D									
Recovery.....	2nd Ventrad series (25V)				2nd Dorsad series (28D)				
Beats.....	10	10	5	= 25	Pause	10	10	8	= 28
Seconds.....	51	51	27	= 2'9"	11"	51	54	40	= 2'25"

*Injury 1: nerves between ganglion and heart severed.

*Injury 2: ganglion extirpated.

TABLE 2

Ascidia mentula no. 16

Graph 16 ¹ . 6.V.13 Pulsation series: 31V-32D-40V-33D.....																
Normal.....	1st Ventrad series (31V)						1st Dorsad series (32D)									
Beats ^a	10	10	10	1	=31	Pause	10	10	10	2	=32	Pause				
Seconds.....	60	57	56	6	=2'59"	10"	58	58	57	13	=3'6"	11"				
	2nd Ventrad series (40D)						2nd Dorsad series (33D)									
Beats ^b	10	10	10	10	=40	Pause	10	10	10	3	=53	Pause				
Seconds.....	57	56	56	56	=3'45"	10"	57	57	56	18	=3'8"	11"				
Graph 16 ² . 7.V.13 Pulsation series: 21V-28D...38V-28D-28V-26D-27V-25D																
Normal.....	1st Ventrad series (21V)						1st Dorsad series (28D)									
Beats ^a	10	10	10	1	=21	Pause	10	10	8	=28	Pause					
Seconds.....	69	64	7		=2'20"	15"	61	61	50	=2'52"	10"					
	Ventrad series (38V)						Dorsad series (28D)									
Beats ^b	10	10	10	8	=38	Pause	10	10	8	=28	Pause					
Seconds.....	54	53	51	43	=3'21"	11"	53	55	44	=2'32'	10"					
Graph 16 ³ . Ventrad series (28V) Dorsad series (26D)																
Beats ^a	10	10	8	=28	Pause		10	10	6	=26	Pause					
Seconds.....	56	53	43	=2'32"	11"		56	52	33	=2'21"	10"					
	Ventrad series (27V)						Dorsad series (25D)									
Beats ^b	10	10	7	=27	Pause		10	10	5	=25	Pause					
Seconds.....	55	55	37	=2'27"	12"		55	56	28	=2'19"	10"					
Graph 16 ⁴ . 7.V.13 Pulsation series: 95D-85V.....																
Ganglion cut out...	Ventrad series (85V)						Dorsad series (95D)									
Beats.....	10	10	10	10	10	=85	Pause	10	10	10	10	10	10	10	10	=95+
Seconds.....	42	41	41	41	43	=5'+	9"	46	48	47	45	45	44	47	=6'	
Graph 16 ⁵ . 8.V.13 Pulsation series: 21V-21D-24V-22D-23V-22D....																
Recovery.....	Ventrad series (21V)						Dorsad series (21D)									
Beats ^a	10	10	1	=21	Pause		10	10	1	=21	Pause					
Seconds.....	70	69	7	=2'26"	15"		66	68	7	=2'21"	10"					
	Ventrad series (24V)						Dorsad series (22D)									
Beats ^b	10	10	4	=24	Pause		10	10	2	=22	Pause					
Seconds.....	67	66	26	=2'39"	14"		64	68	13	=2'25"	10"					
Graph 16 ⁶ . 19.V.13 Pulsation series: 18D-21V-19D...20D-24V																
	Ventrad series (21V)						Dorsad series (19D)									
Beats.....	10	10	1	=21	Pause		10	9	=19	Pause						
Seconds.....	69	67	7	=2'23"	12"		68	61	=2'9"	20"						

TABLE 3
Ascidia mentula, no. 18

Graph 18 ¹ . 21.V.13 Pulsation series: 38D-39V-41D-41V....																		
Normal.....	Ventrads series (39V)					Dorsad series (38D)												
Beats.....	10	10	10	9	=39	Pause	10	10	10	8	=38	Pause						
Seconds.....	53	51	49	50	=3'23"	24"	54	55	53	48	=3'30"	6"						
Graph. 18 ² . 21.V.13 Pulsation series; 54D-54V-80D....																		
Injury 1*.....	Ventrads series (54V)					Dorsad series (54D)												
Beats.....	10	10	10	10	10	4 =54	Pause	10	10	10	10	4 =54	Pause					
Seconds.....	51	50	47	48	49	20 =4'25"	19"	55	55	53	53	25 =3'55"	6"					
Graph 18 ³ . 22.V.13 Pulsation series: 41V-47D-50V-42D....																		
Recovery.....	Ventrads series (41V)					Dorsad series (47D)												
Beats.....	10	10	10	10	1	=41	Pause	10	10	10	10	7 =47	Pause					
Seconds.....	57	55	53	53	7	=3'45"	19"	55	55	53	54	41 =4'18"	9"					
Graph 18 ⁴ . 23.V.13 Pulsation series: 41D-42V-39D-44V....																		
	Ventrads series (42V)					Dorsad series (41D)												
Beats.....	10	10	10	10	2	=42	Pause	10	10	10	10	1 =41	Pause					
Seconds.....	54	53	51	53	11	=3'52"	17"	55	55	53	50	6 =3'39"	6"					
Graph 18 ⁵ . 23.V.13 Pulsation series: 65V-58D-95V.....																		
Injury 2*.	Ventrads series (65V)					Dorsad series (58D)												
Beats.....	10	10	10	10	10	10	5 =65	Pause	10	10	10	10	8 =58	Pause				
Seconds....	50	49	47	46	46	48	24 =5'10"	13"	47	48	47	49	41 =4'41"	8"				
Graph 18 ⁶ . 26.V.13 Pulsation series: 68V-83D..78D-81V..70V-22D-65V-20D..																		
Recovery.	Ventrads series (68V)					Dorsad series (83D)												
Beats...	10	10	10	10	10	10	8 =68	Pause	10	10	10	10	10	10	3 =83	Pause		
Seconds.	51	52	49	48	49	47	41 =5'37"	10"	50	52	50	47	49	48	48	52	17 =6'43"	10"
Graph 18 ⁷																		
Injury 3*	Ventrads series (81V)					Dorsad series (78D)												
Beats...	10	10	10	10	10	10	1 =81	Pause	10	10	10	10	10	10	8 =78	Pause		
Seconds.	45	46	44	44	44	45	45	6 =6'5"	9"	49	50	50	50	47	47	48	41 =6'22"	10"
Graph 18 ⁸																		
Injury 4*.....	Ventrads series (65V)					Dorsad series (22D)												
Beats.....	10	10	10	10	10	10	5 =65	Pause	10	10	2	=22	Pause					
Seconds.....	44	44	42	41	41	43	22 =4'24"	10"	44	50	11	=1'45"	12"					

*Injuries: 1 Incision made severing nerves between ganglion and aboral siphon.

2. Ganglion excised. 3. Pieces sliced off tunic.

4. Oral siphon amputated.

TABLE 4

	GRAPHS	DATE	STATE OF ANIMAL	NUMBER OF BEATS IN VENTRAD SERIES	RANGE OF FLUCTUATION	NUMBER OF BEATS IN DORSAD SERIES	RANGE OF FLUCTUATION
Iamina 15	1	5.V.13	Normal	25,26,26	1	25,26,26	1
	2	8.V.13	Normal	30,42	12	21,30,35	14
	3	8.V.13 <i>a.m.</i>	Nerve severed	57,58	1	126,102	24
	4	8.V.13 <i>p.m.</i>	Recovery	23,25,28	5	22,24,26	4
	5	9.V.13	Recovery	23,25,26	3	20,23,25,25	5
	6	9.V.13	Ganglion extirpated	83—	—	54,68	14
	7	10.V.13	Recovery	21,25	4	26,28	2
Animal 16	1	6.V.13	Normal	31,40	9	32,33....	1
	2,3	7.V.13	Normal	21..38,28,27	17	28..28,28,26,25	3
	4	7.V.13	Ganglion extirpated	85	—	95	—
	5	8.V.13	Recovery	21,24,23	3	21,22,22	2
	6	19.V.13	Recovery	21,24	3	19,20	1
Animal 18	1	21.V.13	Normal	31,40	9	32,33	1
	2	21.V.13	Nerves	54	—	54,80	26
	3		severed				
		22.V.13	Recovery	41,50	9	47,42	5
	4	23.V.13	Recovery	42,44	2	41.39	2
	5	23.V.13	Ganglion extirpated	65,95	30	58—	—
	6	26.V.13	Recovery	68—	•	83—	
	7	26.V.13	Slices off tunic	81		78	
	8	26.V.13	Oral siphons amputated	65—		22—	

TABLE 5
Rate in seconds per consecutive ten beats

	STATE OF ANIMAL	SERIES	ANIMAL NO. 15	ANIMAL NO. 16	ANIMAL NO. 18
1	Normal	Ventrad	55, 55, (29) 58, 55, 53,	60, 59, 56, (6) 57, 56, 56, 56, 69, 64, (7) 54, 53, 51, (43) 56, 5 3, (43) 55, 55, (37)	53, 51, 49, (50)
2	Nerves severed between ganglion and heart	Ventrad	42, 40, 39, 40, 40, (30)	—	51, 50, 47, 48, 49, (20)
3	Recovery	Ventrad	53, 53, (25) 57, 56, (27)	— —	57, 55, 53, 53, (7) 54, 53, 51, 53, (11)
4	Ganglion extirpated	Ventrad	43, 43, 44, 48, 47, 47, 46, 48	42, 41, 41, 41, 43, . . .	50, 49, 47, 46, 46, 48, (24)
5	Recovery	Ventrad	51, 51, 27	70, 69, (7) 67, 66, (26) 69, 67, (7)	
6	Amputation of oral siphon	Ventrad	—	—	—
7	Normal	Dorsad	54, 55, (28) 61, 64, (8)	58, 58, 57, (13) 57, 57, 56, (18) 61, 61, (50) 53, 55, (44) 56, 52, (33) 55, 56, (28)	54, 55, 53, (48)
8	Nerves severed between ganglion and heart	Dorsad	44, 43, 42, 44, 44, 44, 45, 45, 45, 45, 48 (29)	—	55, 55, 53, 53, 53, 54, (25)
9	Recovery	Dorsad	52, 52, (23) 54, 58, (19)	—	55, 55, 53, 54, (41)
10	Ganglion extirpated	Dorsad	43, 44, 43, 41, 42, (19)	46, 48, 47, 45, 45, 45, 44, 47	47, 48, 47, 49, 49, (41)
11	Recovery	Dorsad	51, 54, (40)	66, 68, (7) 64, 68, (13) 68, (61)	50, 52, 50, 47, 49, 48, 48, 52, (17)
12	Oral siphon amputated	Dorsad			44, 50, (11)

Resumen por la autora, M. E. Collett.

La toxicidad de los ácidos en los Infusorios.

II. El papel de la molécula y de los iones.

La adición de ClH a las soluciones de ácidos orgánicos no suministra pruebas concluyentes sobre la toxicidad de la molécula. Las mezclas de ciertos ácidos (fórmico, acético, butírico, valérico, benzóico, y salicílico con *Paramoecium* y *Euplotes*, y las de cítrico con *Euplotes* solamente) con sus sales sódicas son más tóxicas que lo que es de esperar de la pH de la mezcla o de la toxicidad de las sales. Esto parece indicar que las moléculas de estos ácidos son tóxicas. No existe prueba alguna de la toxicidad del anión o de la molécula de los ácidos láctico, 7 succínico o tartárico en la concentración empleada. La toxicidad relativa de las soluciones 0.01 M de las sales sódicas son las siguientes: *Paramoecium*—salicilato, benzoato, tartrato, succinato, citrato, formiato, acetato, butirato, valerianato, cloruro, lactato; *Euplotes*—citrato, solícilato, benzoato, tartrato, succinato, formiato, cloruro, acetato, butirato, valerianato y lactato.

Translation by José F. Nonidez
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THE TOXICITY OF ACIDS TO INFUSORIA

II. THE RÔLE OF MOLECULE AND OF IONS

M. E. COLLETT

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In the literature on the physiological action of acids there is very little definite evidence as to the rôle of the anion and the molecule, although it is clear that the activity of many organic acids is not confined to the H ion. Loeb ('13) states that the power of acids to initiate development in certain unfertilized marine eggs cannot be due to the H ion, for tartaric, citric, and the mineral acids are relatively ineffective as compared with the fatty acids, nor by the anion, for the sodium salts of the efficient acids (butyric, benzoic, etc.) are inadequate. He therefore considers that the action is molecular rather than ionic. Klockmann ('11) compares the disinfecting action of acetic acid alone and mixed with small amounts of HCl to depress its dissociation, and concludes that the molecule is effective as well as the H ion. Van Dam ('18) in studying the souring of whey, finds that the organisms are fairly resistant to the H ion (they continue to grow up to 11.5×10^{-5}) and to Na-lactate (up to 0.1 M), but are very sensitive to the undissociated lactic acid molecule. Thus, when sodium lactate is added to the culture in varying, non-toxic amounts, though the final pH varies from 5.3 to 1.8×10^{-5} , the final concentration of the undissociated lactic acid produced does not vary. Aside from these three papers, I have found no definite discussion of the question.*

In the present series of experiments the problem is approached from two directions and a study is made of the toxicity to *Paramecium* and *Euplotes* of several organic acids used both alone

* Recently Dr. M. H. Jacobs has found that the toxicity H_2CO_3 as compared with other acids is due to the extraordinary ability of its molecule to penetrate living tissue.

TABLE 1

Organic acids alone and combined with HCl

	pH				LENGTH OF LIFE IN MINUTES							
					Paramecium				Euplotes			
	O	A	B	C	O	A	B	C	O	A	B	C
Formic 0.0005 N ¹	3.8	3.7	3.4		1½	2½	1½		10	9	6	
Formic 0.0002 N.....	3.9	3.8	3.7		4	4	4		15	13	12	
Acetic 0.0005 N.....	4.2	4.1	4.0		7½	7	5		35	33	33	
Acetic 0.0002 N.....	4.2	4.1	3.9	3.9	10	11	8	6	40	40	30	22
Propionic 0.0002 N.....	4.3	4.2	4.1	3.9	14	16	12	5	70	70	85	25
Butyric 0.0005 N.....	4.1	4.0	3.9		7	7	6		28	27	23	
Butyric 0.0002 N.....	4.2	4.1	4.0	3.8	13	12	8	6	60	50	35	17
Valeric 0.0005 N.....	4.2	4.0	3.9		7	6	6		29	25	23	
Valeric 0.0002 N.....	4.2	4.1	4.0	3.9	13	13	12	5	50	60	45	19
Caproic 0.0005 N.....	4.1	4.0	3.9		7	8	7		28	30	30	
Caproic 0.0002 N.....	4.2	4.1	4.0	3.9	12	17	9	6	55	55	60	19
Benzoic 0.0005 N ²	3.7	3.6		3.6	3	2	2		2	2	2	
Benzoic 0.0002 N.....	4.3	4.1		3.7	10	6	5	3	20	9	8	6
Salicylic 0.00015.....	4.7		4.4		2	3	4		6	7	6	
Lactic 0.0001 N.....	4.2	3.9	3.6	3.5	8	6	4	2	10	8	8	8
Tartaric 0.0001 N.....	4.0	3.9	3.5	3.5	8	5	3	2	10	7	8	5
Citric 0.0002 N.....	4.1	4.0	3.9	3.6	15	6	3	3		8	8	8
Citric 0.0001 N.....	4.5	4.3	4.0	3.7	70	7	6	5	120	20	8	6
Lactic 0.0002 N.....	3.7	3.5	4.5	3.2	2	2	2	2	6	6	6	5

	pH				LENGTH OF LIFE			
					Paramecium		Euplotes	
HCl ²	3.24				2		4	
	3.54				2		5	
	3.85				3		5	
	4.14				4		7	
	4.14				9		12	
	4.74				20		170	
	5.05				35		180	
	5.6				40		180	
	6.6				120		20h	
	7.0							
	7.7				140		20h	
	8.4				25		18h	
	8.5				—		170'	

¹ Formic to caproic, organisms washed in pond-water.² Benzoic to lactic, organisms washed in distilled water.

and in conjunction either with their sodium salts or with HCl. The technique is substantially the same as that reported in my first paper. The only differences are that the organisms were washed in twice-distilled water instead of in pond-water, and that the test-tubes were of Pyrex instead of ordinary soft glass. Ordinary distilled water is very toxic, but the toxicity very largely disappears after a second distillation over glass from acid solution (Lyon). The time at which two-thirds of the organisms ceased to move their cilia was taken as the average time of death. The length of life observed in any solution would of course be the reciprocal of the toxicity.

To 100 cc. portions of the organic acid solution one or more drops of 0.1 N HCl were added and the pH of each portion was determined by means of indicators. The toxicity of the mixtures was then tested and compared with HCl of the same pH and with the organic acid alone. In the preliminary experiments the procedure was to test the different acids on successive days, first without HCl, next day with one drop of HCl, then with two drops, and so on. This is a fairly satisfactory method if the results are checked by frequent repetition at considerable intervals. Further study has shown that *Euplotes* is apt to vary in resistance most unexpectedly, especially in late winter; consequently it is a better procedure to test all of the mixtures simultaneously and so under precisely the same conditions.

In the preliminary experiments ('19) the addition of one or two drops of HCl seemed to lessen the toxicity to *Euplotes* of several of the acids. This result, however, is not obtained if the day-to-day variation is eliminated by testing the mixture simultaneously. The results are given in table 1. The addition of one or even two drops of HCl, although it increases the pH of the mixture somewhat and must force the already very slightly dissociated organic acid almost wholly into molecular form, leaves the toxicity of the mixture almost unchanged. A further addition of HCl produces a marked increase in toxicity which then follows closely the curve for HCl alone. The fact that toxicity is not increased by the first addition of HCl indicates that such a mixture is a trifle less toxic as compared with HCl than is

the organic acid alone. This might be interpreted as evidence of the toxicity of the organic anion, which would be removed as ionization of the organic acid was depressed by the addition of HCl. An equally plausible interpretation would be that the organic molecule itself (present in considerable amount before the addition of HCl) is toxic, and that it is antagonized up to a certain point by the increasing acidity of the mixture. Without further evidence it is difficult to decide which interpretation is most nearly correct. Some preliminary experiments with xylol indicate that the addition of a small quantity of HCl has a distinctly antitoxic influence. A slight increase in pH may thus under certain conditions have a protective effect. In this respect the H ion acts similarly to other cations in certain forms of salt antagonism (Lillie, '12, '13 Osterhout, '18).

Xylol 0.6 saturated solution in water

	LENGTH OF LIFE IN MINUTES		
	Alone	HCl 1 dr.	HCl 2 dr.
Paramecium.....	1	2	8
Euplotes	20	20	35

A second means of approach is at hand in a study of the relative toxicity of the organic acids alone and when combined with their sodium salts.

The toxicity of the different sodium salts of the organic acids when used alone varies somewhat at different concentrations, as is indicated in the following series (table 2).

0.1 M

Paramecium: Benzoate = citrate = salicylate > tartrate > succinate > formate—valerianate > chloride > lactate.

Euplotes: Benzoate = citrate > salicylate = tartrate = chloride > butyrate > valerianate > formate = lactate > succinate > acetate.

0.01 M

Paramecium: Salicylate = benzoate = tartrate = succinate > citrate > formate-valerianate = chloride = lactate.

Euplotes: Citrate > salicylate > benzoate = tartrate > succinate > formate—valerianate = lactate. Here as in many other physiological reactions (*Höber, True*) the order of toxicity is: benzoate > salicylate > tartrate > formate-valerianate > chloride. In spite of the general similarity between the order for *Paramecium* and that for *Euplotes*, there are several important differences. *Euplotes*, so strikingly resistant to pH, falls to the level of *Paramecium* in resistance to concentrated solutions of several of the salts, e.g., chloride, formate, butyrate, benzoate, salicylate, and citrate.

Although all of these salts are fairly toxic at concentrations of 0.5 M and above, very few of them remain so at lower concentrations. At 0.01 M only salicylate, citrate, benzoate, and tartrate kill *Euplotes* within sixty minutes. The extraordinary toxicity of Na-citrate must be due to the anion rather than to the cation, for Na-citrate 0.01 M is more toxic to *Euplotes* than NaCl of five times its concentration. At still lower concentrations, such as would be present in acids of 0.001 N or less, only three anions would still be significantly toxic—salicylate and citrate to *Euplotes*, salicylate to *Paramecium*. Consequently, we cannot explain the extraordinary toxicity of the monobasic fatty acids and cyclic acids as due to the toxic action of their anions, except perhaps to some extent in the case of salicylic.

In order to test the point further and to secure additional evidence as to the activity of the molecule, observations were made with mixtures of each acid with its sodium salt (table 2).

With salicylic in the case of *Paramecium* and with salicylic, benzoic, and citric in the case of *Euplotes* the salt-acid mixture is more toxic than the acid alone, even though the salt is relatively non-toxic. Since ionization is depressed in the salt-acid mixture and the number of acid molecules is consequently increased, the greater toxicity of the salt-acid mixture must be due to the activity of the molecule. It is noteworthy that these three acids are among the most toxic of those studied. With these three exceptions, the addition of 0.01 M Na-salt decreases the toxicity of all the acids tested. With higher concentrations of salt there is generally a further decrease in toxicity, which, how-

ever, is limited by the increasing toxicity of the salt itself. The decrease in toxicity in the acid-salt mixtures is of course due largely to diminished pH.

It is interesting to note that the decrease in many cases is less than would be expected. Thus, in formic acid plus 0.05 M

TABLE 2
Toxicity of Na salts with and without their acids

Concentration of salt.....	LENGTH OF LIFE IN MINUTES										
	Paramecium				Euplotes				pH		
	M 0.0	0.01	0.05	0.1	0.0	0.01	0.05	0.1	0.01	0.05	0.1
<i>Salt alone</i>											
Na Cl.....		15h	120	90		15h	25	8			
Na formate.....		12h	70	40		12h	60	45			
Na acetate.....		15h	60	25		8h	180	150	6.7	7.1	7.2
Na butyrate.....		12h	90	15		12h	15	16	6.7	7.3	7.3
Na valerianate.....		15h	20	10		15h	40	35			
Na benzoate.....		22'	9	1		55'	1	1			
Na salicylate.....		15	10	2		25	10	7			
Na succinate.....		40	80	15		120	90	70			
Na lactate.....		19h	19h	120		19h	4h	50			
Na tartrate.....		40	10	3		60	35	10			6.5
Na citrate.....		180	6	1		10	4	2	7.3	8.2	8.2
<i>Acid and its salt</i>											
HCl 0.00025 N....	4	7	11	9	8	7	8	8	4.0	4.1	4.1
Formic 0.0005 N...	2	9	14	20	6	8	7	5	5.1	5.6	5.9
Acetic 0.001 N....	3	25	20	65	12	50		90	6.4	6.5	6.6
Butyric 0.001 N...	4	30	43	45	8	80	150	110	5.8	6.5	6.5
Benzoic 0.0005 N..	2	6	4	2	2	1	1	1	5.1	5.5	5.5
Salicylic 0.0002 N..	2	1	1	1	5	1	1	1	4.4	5.1	5.1
Lactic 0.0005 N....	3	24h	24h	60	10	24h	24h	60	7.0	7.2	7.2
Tartaric 0.0005 N..		10	10	10	8	20	55	25	4.4	4.9	6.5
Citric 0.0005 N....	2	120	6	2	10	3	3	3	7.0	7.2	7.2
Succinic 0.0005 N..	2	60	90	12	8		120	60	6.5	6.4	6.6
Valeric 0.001 N....		14	25	107	8	16	35	7	5.5	5.6	5.1

Na-formate Euplotes lives eight minutes, although in Na-formate alone it lives sixty minutes and in HCl (of the same pH as the mixture) 80 minutes. Other examples are given in the table 3. The length of life in acid plus salt of non-toxic concentration is less than the pH would indicate in the case of all the monobasic

fatty acids tested. Here the molecule must be capable of toxic action. In the case of formic acid this would ordinarily be insignificant on account of the almost complete dissociation of the acid, but with other members of the series (acetic to caproic) it is important and accounts very largely for their great toxicity as compared with HCl of the same pH. With benzoic, although the salt is somewhat toxic to *Paramecium*, the toxicity of the acid-salt mixture is too great to be due to the action of the anion or to the pH of the mixture, and must therefore be due in part to the acid molecule.

TABLE 3

	LENGTH OF LIFE IN MINUTES							
	Acid alone		Acid and salt		Salt 0.01 M		HCl pH of acid-salt	
	Para- mecium	Eu- plotes	Para- mecium	Eu- plotes	Para- mecium	Eu- plotes	Para- mecium	Eu- plotes
Formic.....	2	6	9	8			40	180
Acetic	3	12	50	90	15h	8h	100	180
Butyric	4	8	30	65			40	180
Valeric.....	4	8	14	25			40	180
Benzoic.....	2	2	6	1	22	55	35	180
Salicylic	2	5	1	1	15	25	9	12
Citric.....	2	10	120	3	180	10		
Succinic.....	2	8	60	120	40	120	120	20h
Tartaric	2	8	10	20	30	60	9	12
Lactic.....	3	10						

The anion of citric acid is only slightly toxic to *Paramecium*, the molecule not at all. The same is true of succinic and as regards both organisms. With lactic and tartaric the length of life is about the same as in HCl of the same pH.

It is possible that what seems to be the toxic action of the molecule may instead be due to increased sensitiveness of the organism to the salt when in the presence of acid. The simpler and more probable explanation, however, is the one developed here.

SUMMARY

1. When ionization of these organic acids is depressed by the addition of HCl, the toxicity of the mixture is at first unchanged, but with further addition of HCl is increased.

2. The fact that the toxicity of the mixture is unchanged by a small addition of HCl, although the pH is slightly increased, may be due either to the disappearance of the toxic organic anion or to the partial antagonism of the molecule by HCl.

3. Of the acids studied, only salicylic, benzoic, and citric have anions which are toxic in concentrations of 0.01 M or less, as judged by the behavior of their sodium salts.

4. The relative toxicity of the sodium salts 0.01 M is:

Paramecium: salicylate > benzoate > tartrate = succinate > citrate > formate = valerianate = chloride = lactate.

Euplotes: citrate > salicylate > benzoate = tartrate > succinate > formate = valerianate = lactate.

5. Mixtures of certain acids (formic, acetic, butyric, valeric) with non-toxic concentrations of their salts are more toxic than can be accounted for by the pH of the mixtures. Benzoic and salicylic (with both organisms) and citric (with Euplotes) when mixed with a dilute solution of their Na-salts are also more toxic than can be accounted for by the pH or by the toxicity of the salts. This evidence seems to indicate that the molecules of such acids are in themselves toxic.

6. There is no evidence of toxicity on the part of anion or molecule of lactic, succinic or tartaric acids at the concentrations used.

It is a pleasure to record my indebtedness to Dr. R. S. Lillie, in whose laboratory at Clark University this work was done, for his help and interest in the problem.

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Resumen por la autora, M. E. Collett.

La toxicidad de los ácidos en los Infusorios.

III. Antagonismo de la acción tóxica de los ácidos por medio de los cloruros inorgánicos.

La duración de la vida en soluciones de avrios ácidos se prolonga considerablemente mediante acidión de cloruros inorgánicos. Los ácidos probados pueden distribuirse en dos grupos: E primero comprende el ClH, y los ácidos fórmico, acético, succínico, láctico, tartárico y cítrico; el segundo comprende los ácidos butírico, caprónico, benzoico, salicílico y fenilacético. El poder antagonista relativo de los cloruros es:

	<i>Ácidos del grupo I</i>	<i>Ácidos del grupo II</i>
Para-	} Ca>Sr>Ba≥Mg Mg>Co>Mn>Cd=Ni=Zn	Ca>Sr>Ba>Mg
moecium		Mg>Mn>Co>Cd>Ni-Zn
Euplotes	} Ca>Ba>Sr>Mg Mg>Mn>Co>Cd>Ni=Zn	Ca>Sr>Ba>Mg
		Mg>Mn>Cd>Co>Ni=Zn

El ClNa aumenta el poder antagonista de los Cl₂Ba y Cl₂Mg contra todos los ácidos pero el de los Cl₂Ca y Cl₂Sr solamente contra los ácidos del segundo grupo. La acción de las sales es probablemente doble. Cada una de ellas tiene alguna influencia sobre la reversión de las fases y de este modo también sobre la rapidez de la penetración del ácido, y cada una de ellas estabiliza también los coloides celulares contra el hinchamiento o acción coagulante del ión H.

Translation by José F. Nonidez
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THE TOXICITY OF ACIDS TO INFUSORIA

III. ANTAGONISM OF THE TOXIC ACTION OF ACIDS BY INORGANIC CHLORIDES

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TWO FIGURES

As a means of determining the way in which acids act in attacking the living cell, the following experiments have been made in which the acids are combined with varying amounts of certain inorganic chlorides. The experiments were performed at Clark University and were undertaken at the suggestion of Dr. R. S. Lillie, for whose help and interest I am exceedingly grateful. The acids studied are as follows:

Inorganic: HCl.

Monobasic fatty: formic, acetic, butyric, caproic.

Cyclic: benzoic, salicylic, phenylacetic, succinic.

Hydroxy: lactic, tartaric, citric.

The concentrations chosen vary from acid to acid, but in every case are such as will kill *Paramecium* in about two minutes and *Euplotes* in less than twenty. The acids were tried alone and in combination with the following chlorides:

<i>Alkali:</i> NH_4Cl , NaCl , KCl .	0.01	–0.1	M
<i>Alkaline-earth:</i> BaCl_2 , CaCl_2 , MgCl_2 , SrCl_2 .	0.001	–0.01	M
<i>Heavy metal:</i> CdCl_2 , CoCl_2 , MnCl_2 .	0.001	–0.01	M
NiCl_2 , ZnCl_2 .	0.0001	–0.001	M

The range of salt concentrations was selected to include not only the optimal concentration, but also higher and lower concentrations which are less efficient. The results are not the same with all the acids, but in a general way two main groups are defined of which acetic and caproic are types.

EXPERIMENTAL EVIDENCE

The toxicity of acetic acid is considerably lessened by the addition of appropriate amounts of the chlorides (table 1). Among the chlorides of the alkali metals the antagonism is greatest with NaCl and least with NH_4Cl (which in itself is very toxic). The higher concentrations are especially effective with Paramecium; with Euplotes they are very toxic, although

TABLE 1

ACETIC ACID 0.001 N	LENGTH OF LIFE IN MINUTES									
	Paramecium					Euplotes				
	0.0	0.005	0.01	0.05	0.1	0.0	0.005	0.01	0.05	0.1
Concentration of salts M.	0.0005	0.0005	0.001	0.005	0.01	0.0	0.0005	0.001	0.005	0.01
Monovalent cations.....										
Bivalent cations.....										
Alone.....	3					12				
Plus NaCl.....		6	8	12	11		15	30	7	7
Plus KCl.....		2	2	5	5		20	15	7	7
Plus NH_4Cl		3	3	4	4		6	5	4	4
Plus BaCl_2			8	8	10			40	70	80
Plus CaCl_2		25	35	65	70			63	90	120
Plus MgCl_2			4	8	9			20	40	30
Plus SrCl_2			3	25	40			40	40	50
Plus BaCl_2 plus NaCl....			6	15	10			80	180+	180+
Plus CaCl_2 plus NaCl....		45	50	90	50			160	180+	180+
Plus MgCl_2 plus NaCl....			6	12	7			15	180+	180+
Plus SrCl_2 plus NaCl....			10	35	20			85	5	5
Plus CdCl_2			<1	<1	<1			7	10	10
Plus CoCl_2			2	3	3			4	6	7
Plus MnCl_2			3	7	9			6	15	18
Plus NiCl_2		1	<1				4	4		
Plus ZnCl_2		1	<1				4	4		

lower concentrations are fairly efficient. This is to be correlated with the fact that Euplotes is much less resistant than Paramecium to NaCl alone, at least in concentrations of 0.05 M and above although this is not the case at 0.01 M. At the optimal concentration the length of life is increased four times for Paramecium and two or three times for Euplotes by the addition of NaCl to the acid.

The efficiency of the alkaline earth chlorides is as follows:

Paramecium: $\text{Ca} > \text{Sr} > \text{Ba} = \text{Mg}$

Euplotes: $\text{Ca} > \text{Ba} > \text{Sr} > \text{Mg}$

The antagonistic power of this group is on the whole greater than that of NaCl as regards acetic acid. Thus the length of life is multiplied as follows:

	<u>Ca</u>	<u>Sr</u>	<u>Ba</u>	<u>Mg</u>	<u>Na</u>
Paramecium:	$23 \times$	$15 \times$	$3 \times$	$3 \times$	$4 \times$
Euplotes:	$10 \times$	$4 \times$	$7 \times$	$3 \times$	$2\frac{1}{2} \times$

The chlorides of the heavy metals are rather less efficient antagonists, partly because of their greater toxicity. The order of antagonistic power is:

Paramecium: $\text{Mg} > \text{Mn} > \text{Co} > \text{Cd} = \text{Ni} = \text{Zn}$

Euplotes: $\text{Mg} > \text{Mn} > \text{Co} > \text{Cd} > \text{Ni} = \text{Zn}$

When used alone their relative toxicity is (table 2)

Paramecium: $\text{Mg} < \text{Co} < \text{Mn} < \text{Cd} < \text{Ni} < \text{Zn}$

Euplotes: $\text{Mg} < \text{Mn} < \text{Co} < \text{Cd} < \text{Ni} < \text{Zn}$

If the pure salts are combined with NaCl in the ratio of one to ten molar, their toxicity is in most cases considerably diminished, although the general relations are similar. There are a few exceptions: the toxicity of BaCl_2 to Paramecium is unchanged and that of SrCl_2 , CdCl_2 and CoCl_2 is noticeably increased by such an addition. The order of toxicity of the pure salts given above agrees only in part with those given for Paramecium by McClelland and Peters⁴⁰ and by Woodruff.⁴²

Woodruff $\text{K} < \text{Zn} < \text{Sr} < \text{Mg} < \text{Mn} < \text{Co} < \text{Ni} < \text{Cd} < \text{H}$

McClelland and Peters $\text{Ba} < \text{Mg} < \text{Ni} < \text{Co} < \text{Zn} < \text{Mn}$

In all these lists, however, Cd and Mn are among the most toxic, Ni and Zn are at certain concentrations very toxic, and Co occupies an intermediate position. The lack of agreement is due in part to differences in technique and to the concentrations studied and probably also to race and species differences.

If, instead of adding a single salt to the acid, we add both an alkaline earth chloride and NaCl, in the ratio of one molecule of

the former to ten of the latter, there is a marked change in anti-toxic action. With most of the alkaline earth chlorides antagonistic power may be increased 50 per cent or more by the addition of NaCl; the amount of increase varies with the concentration of the salts. For example, with *Paramecium* the power of BaCl₂

TABLE 2

SALTS ALONE	LENGTH OF LIFE IN MINUTES							
	Paramecium				Euplotes			
	0.0001 0.0001	0.01 0.001	0.05 0.005	0.1 0.01	0.1 0.0001	0.01 9.001	0.05 0.005	0.1 0.01
Concentration of salts M. Monovalent cations..... Bivalent cations.....								
NaCl.....		15h	2h	40'		15h	25'	8'
KCl.....								
NH ₄ Cl.....								
BaCl ₂				25				<90
CaCl ₂				90+				90+
MgCl ₂				10				90
SrCl ₂				100+				90
BaCl ₂ plus NaCl.....				20				90+
CaCl ₂ plus NaCl.....				100				90+
MgCl ₂ plus NaCl.....				40				10
SrCl ₂ plus NaCl.....				100+				5
CdCl ₂			<1	<1			20	20
CoCl ₂			6	6			35	30
MnCl ₂			3	2			90+	120+
NiCl ₂	3	< 1			25	15		
ZnCl ₂	1	< 1			12	11		
CdCl ₂ plus NaCl.....			1	1			11	5
CoCl ₂ plus NaCl.....			15	8			9	7
MnCl ₂ plus NaCl.....			25+	25			90	90
NiCl ₂ plus NaCl.....	25	9			5	<5		
ZnCl ₂ plus NaCl.....	7	7			<5	<5		

0.005 M to antagonize the toxic action of acetic is increased 100 per cent by the addition of NaCl; but the addition of NaCl does not in any way improve the antitoxic power of BaCl₂ at other concentrations (0.001 or 0.01 M). Similarly, the antitoxic power of CaCl₂ and SrCl₂ at 0.001 or 0.005M is decidedly increased

by the addition of NaCl, but at 0.01 M is decreased. With Euplotes the addition of NaCl to the alkaline earth chlorides increases their antitoxic action somewhat at all concentrations, except in the case of SrCl_2 : here it increases the antitoxic action of SrCl_2 , 0.001 M, but decreases that of SrCl_2 0.01 M.

The same general results are obtained with formic acid (table 8). With butyric and caproic, however, somewhat different results appear, especially in the mixtures containing NaCl. The addition of NaCl diminishes the toxicity of butyric acid to

TABLE 3

BUTYRIC 0.001 N. Concentration of salts M. Monovalent cations Bivalent cations	LENGTH OF LIFE IN MINUTES									
	Paramecium					Euplotes				
	0.0	0.005 0.0005	0.01 0.001	0.05 0.005	0.1 0.01	0.0	0.005 0.0005	0.01 0.001	0.05 0.005	0.1 0.01
Alone	4					8				
Plus NaCl			10	12	12			20	80+	80
Plus BaCl_2			7	10	9			40	60	70
Plus CaCl_2			50	70	65			70	90	120
Plus MgCl_2			8	11	7			12	15	23
Plus SrCl_2			10	25	30			40	50	45
Plus BaCl_2 plus NaCl. . .			8	35	25			80	180+	180+
Plus CaCl_2 plus NaCl. . .			60	80	60			120	180+	180+
Plus MgCl_2 plus NaCl. . .			15	15	15			60	180+	180+
Plus SrCl_2			15	45	30			120	180+	180+

Paramecium as it does in the case of acetic. With Euplotes NaCl is highly toxic if used alone or in conjunction with acetic, but when added to butyric it becomes an efficient antagonist and increases the length of life ten times (table 3). Caproic (table 4) is more difficult to antagonize but even so NaCl 0.1 M decreases toxicity to Euplotes by three-fourths instead of increasing it as it does with acetic. The efficiency of the alkaline earth chlorides is:

Paramecium: $\text{Ca} > \text{Sr} > \text{Ba} = \text{Mg}$

Euplotes: $\text{Ca} > \text{Sr} > \text{Ba} > \text{Mg}$

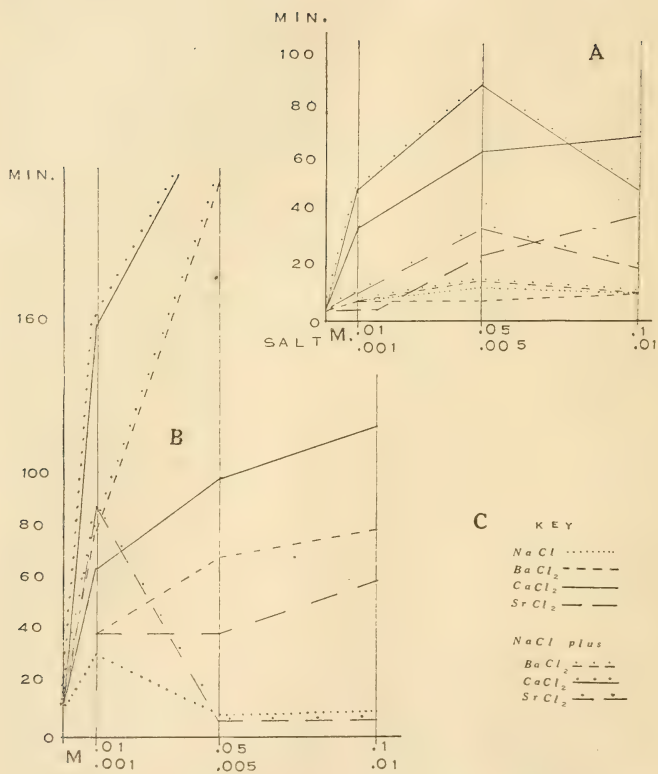


Fig. 1 Acetic acid with and without salts. A, Paramecium; B, Euplotes; C, key to symbols.

just as is the case with acetic. When these chlorides are combined with NaCl, even at the highest concentration used, their protective power for both organisms against caproic or butyric is increased many-fold, while with acetic this was true only for *Paramecium*. Thus *Euplotes* lives eight minutes in caproic

TABLE 4

CAPROIC 0.001 N	LENGTH OF LIFE IN MINUTES									
	Paramecium					Euplotes				
	0.0	0.005	0.01	0.05	0.1	0.0	0.005	0.01	0.05	0.1
Monovalent cations.....	0.0	0.0005	0.001	0.005	0.01	0.0	0.0005	0.001	0.05	0.1
Bivalent cations.....										
Alone.....	5					10				
Plus NaCl.....			7	8	10			16	25	40
Plus BaCl ₂			5	5	5			30	30	30
Plus CaCl ₂			10	18	30			40	40	60
Plus MgCl ₂			4	5	5			14	15	11
Plus SrCl ₂			5	5	8			20	20	25
Plus BaCl ₂ plus NaCl.....			7	8	7			35	80	60
Plus CaCl ₂ plus NaCl.....			20	35	25			60	150	100
Plus MgCl ₂ plus NaCl.....			8	10	9			15	80	60
Plus SrCl ₂ plus NaCl.....			8	18	13			45	120	100
Plus CdCl ₂			<1	<1	<1			5	4	6
Plus CoCl ₂			3	4	4			5	4	5
Plus MnCl ₂			3	5	6			5	6	12
Plus NiCl ₂		<1	<1				4	5		
Plus ZnCl ₂		<1	<1				4	5		
Plus CdCl ₂ plus NaCl.....			6	8	4			7	15	18
Plus CoCl ₂ plus NaCl.....			7	11	8			7	55	50
Plus MnCl ₂ plus NaCl.....			7	20	11			8	180	90
Plus NiCl ₂ plus NaCl.....		2	3				4	5		
Plus ZnCl ₂ plus NaCl.....		2	3				7	10		

acid alone, forty-five minutes in acid plus SrCl₂ 0.01 M, and 180 minutes in acid plus SrCl₂ 0.01 M plus NaCl 0.1 M. As to the heavy metals, the order of efficiency with caproic is for both organisms: Mg > Mn > Co > Cd = Ni = Zn. This agrees exactly with the order observed with acetic. When combined with NaCl the antitoxic efficiency of all these chlorides is greatly in-

creased. This is interesting, for, when the acid is omitted and the salts are used alone, the addition of NaCl increases the toxicity of CdCl_2 and CoCl_2 to Euplotes, just as it does with SrCl_2 .

Turning, now, to the cyclic acids (tables 5, 9, 10), we find much the same condition of affairs as with caproic, and like caproic they are more difficult to antagonize than formic, acetic, or butyric. These same lipid-soluble acids were found by Harvey³⁸

TABLE 5

BENZOIC 0.0005 N	LENGTH OF LIFE IN MINUTES									
	Paramecium					Euplotes				
	0.0	0.005 0.0005	0.01 0.001	0.05 0.005	0.1 0.01	0.0	0.005 0.0005	0.01 0.001	0.05 0.005	0.1 0.01
Concentration of salts M.										
Monovalent cations.....										
Bivalent cations.....										
Alone.....	2					2				
Plus NaCl.....			5	10	10			20	20	25
Plus KCl.....			3	4	5			4	7	10
Plus BaCl ₂			4	7	6			10	12	17
Plus CaCl ₂			10	20	30			15	23	33
Plus MgCl ₂			5	7	6			5	5	4
Plus SrCl ₂			4	7	10			15	15	20
Plus BaCl ₂ plus NaCl.....			5	10	10			18	40	35
Plus CaCl ₂ plus NaCl.....			13	25	20			25	60	60
Plus MgCl ₂ plus NaCl.....			5	7	6			5	5	4
Plus SrCl ₂ plus NaCl.....			8	14	17			28	45	40
Plus CdCl ₂			2	5	2			2	3	5
Plus CoCl ₂			2	4	3			2	3	3
Plus MnCl ₂			3	7	6			3	3	8
Plus NiCl ₂		7	5				12	30		
Plus ZnCl ₂		7	5				20	30		

and by Crozier³⁶ to penetrate living tissue most readily, and by Loeb to be the most efficient in inducing artificial parthenogenesis. In many physiological processes it is almost impossible to reverse or protect against the action of cyclic compounds (Höber).²⁴ If the cyclic acid is mixed with NH_4Cl there is little decrease in toxicity, for the salt itself is deadly. With KCl there is a fair amount of antagonism, and with NaCl still more; the antagonistic power of both increases with the concentration, even for Euplotes.

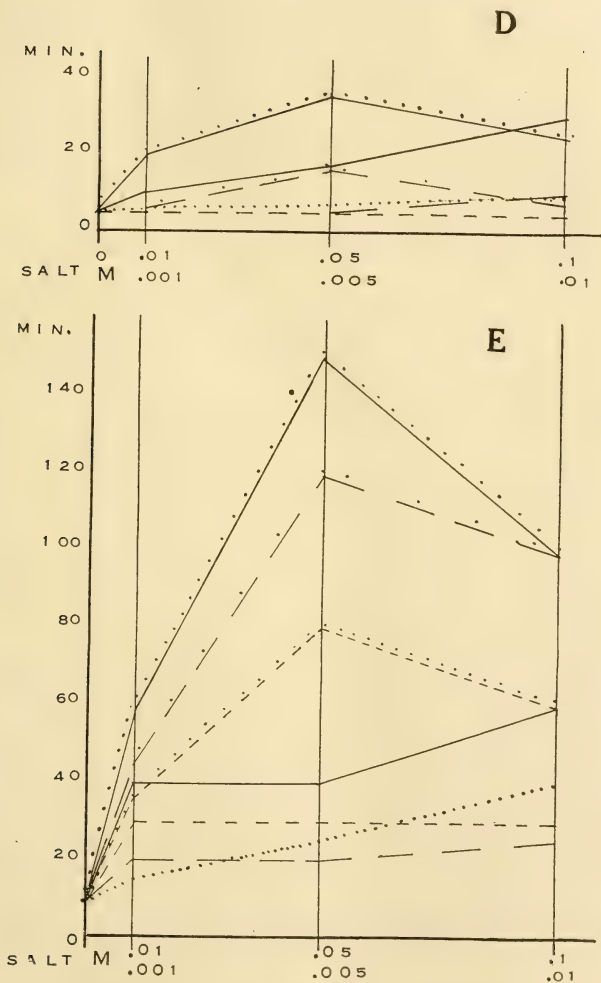


Fig. 2 Caproic acid with and without salts. D, *Paramecium*; E, *Euplotes*.

The efficiency of the alkaline earth chlorides here as with caproic is:

Paramecium: $\text{Ca} > \text{Sr} > \text{Ba} > \text{Mg}$

Euplotes: $\text{Ca} > \text{Sr} > \text{Ba} > \text{Mg}$

When NaCl is added to the mixture of acid and alkaline earth chloride there is at all concentrations a further decrease in toxicity as in the case of caproic. The heavy metal chlorides are even less efficient, although the same order holds as with caproic and acetic; here, too, there is an increase in antagonistic power with the addition of NaCl.

TABLE 6

SUCCINIC 0.0005 N	LENGTH OF LIFE IN MINUTES									
	Paramecium					Euplotes				
	0.0	0.005	0.01	0.05	0.1	0.0	0.005	0.01	0.05	0.1
Concentration of salts M.	0.0005	0.0005	0.001	0.005	0.01	0.0095	0.001	0.005	0.01	0.01
Monovalent cations.....										
Bivalent cations.....										
Alone	2					8				
Plus NaCl			15	18	10			15	15	10
Plus KCl			8	7	5			15	18	22
Plus NH ₄ Cl			6	4	3			15	4	3
Plus BaCl ₂			10	12	15			120		100
Plus CaCl ₂			50	70	80			180		180
Plus MgCl ₂			10	11	10			20		35
Plus SrCl ₂			18	30	45			120		40
Plus BaCl ₂ plus NaCl....			14	30	18			120+	180+	120
Plus CaCl ₂ plus NaCl....			90	180	120			120+	180+	4h
Plus MgCl ₂ plus NaCl....			10	15	15			80	180+	180+
Plus SrCl ₂ plus NaCl....			30	120	25			130+	10	10

These results hold for benzoic, salicylic, and phenylacetic, but for succinic (table 6) we must turn to HCl (table 7) or to tartaric and the other hydroxy-acids (tables 11, 12, 13) for a parallel. The addition of NaCl to a hydroxy-acid may increase or leave unchanged its toxicity to Euplotes at the same time that it protects Paramecium in the usual way, just as was the case with acetic. The efficiency of the alkaline earth chlorides is for both organisms, $\text{Ca} > \text{Sr} > \text{Ba} > \text{Mg}$. This differs slightly from the order with acetic (Paramecium, $\text{Ca} > \text{Sr} > \text{Ba} = \text{Mg}$;

Euplotes, $\text{Ca} > \text{Ba} > \text{Sr} > \text{Mg}$), but agrees with that found for caproic. When NaCl is added to the mixture of alkaline earth chloride and acid, the toxicity of the mixture is greatly diminished, except in the case of Sr where it is markedly augmented, and this increase holds for the more dilute as well as for the more concentrated salt solutions. The order of efficiency in antitoxic power for the heavy metal chlorides is:

Paramecium: $\text{Mn} > \text{Mg} > \text{Co} > \text{Cd} > \text{Ni} > \text{Zn}$

Euplotes: $\text{Mn} > \text{Mg} > \text{Cd} > \text{Co} > \text{Ni} > \text{Zn}$

This varies only slightly from the order observed for butyric and acetic.

To summarize:

1. The acids tested fall into two groups. The first group includes HCl, formic, acetic, succinic, lactic, tartaric, citric. The second, the more lipid soluble group, includes butyric, caproic, benzoic, salicylic, phenylacetic.

2. NaCl diminishes the toxicity of all the acids to Paramecium. For Euplotes it is similarly efficient at all concentrations between 0.01 and 0.1 M with acids of the second group, but only at low concentrations (0.01 to 0.05 M) with acids of the first group.

3. The relative antagonistic power of the alkaline earth chlorides is:

Acids of group 1

Acids of group 2

Paramecium: $\text{Ca} > \text{Sr} > \text{Ba} \geq \text{Mg}$

$\text{Ca} > \text{Sr} > \text{Ba} > \text{Mg}$

Euplotes: $\text{Ca} > \text{Ba} \geq \text{Sr} > \text{Mg}$

$\text{Ca} > \text{Sr} > \text{Ba} > \text{Mg}$

4. The addition of NaCl, in a ratio of ten to one molecular, to the alkaline earth chlorides increases their antitoxic power toward acids of both groups, with the following exceptions:

Paramecium: antagonistic power of 0.01 M CaCl_2 and SrCl_2 is diminished with acids of the first group and left unchanged with acids of the second group.

Euplotes: antagonistic power of SrCl_2 0.005 and 0.1 M is greatly diminished with acids of the first group.

5. The antagonistic power of the heavy metal chlorides is:

Acids of group 1:

Paramecium: $\text{Mg} > \text{Co} > \text{Mn} > \text{Cd} = \text{Ni} = \text{Zn}$

Euplotes: $\text{Mg} > \text{Mn} > \text{Co} > \text{Cd} > \text{Ni} = \text{Zn}$

Acids of group 2:

Paramecium: $\text{Mg} > \text{Mn} > \text{Co} > \text{Cd} > \text{Ni} = \text{Zn}$

Euplotes: $\text{Mg} > \text{Mn} > \text{Cd} > \text{Co} > \text{Ni} = \text{Zn}$

6. Antagonistic power is somewhat limited by the toxicity of the salts concerned. At a concentration of 0.01 M the toxicity of the pure salts is:

Paramecium: $\text{Ca} < \text{Sr} < \text{Mn} < \text{Ba} < \text{Mg} < \text{Co} < \text{Cd} < \text{Ni} = \text{Zn}$

Euplotes: $\text{Ca} < \text{Sr} < \text{Mn} < \text{Mg} < \text{Ba} < \text{Co} < \text{Cd} < \text{Ni} < \text{Zn}$

When combined with NaCl, toxicity is reduced for all except Sr, Cd and Co (Euplotes).

VISIBLE CHANGES IN THE PROTOPLASM

The visible effects produced by the action of the acids alone and when combined with salts afford some interesting contrasts. Acids like acetic when used alone produce a rigidity of the cilia accompanied by swelling and finally disintegration. The cell body assumes a coarsely granular appearance just before the cilia stop moving and the vacuoles grow large and rigid. Rifts appear in the protoplasm due to the clumping of particles and the consequent squeezing out of fluid. If the acid is more dilute than the solutions used in the present experiments, the vacuoles soon cease to pulsate and like the rest of the body swell enormously. The more concentrated the acid the less the swelling. When salts are added, results vary with the concentration of the salt. With the more dilute salt solutions there is a great swelling followed by coagulation and death. In mixtures containing slightly more salts the coagulation is greatly delayed or even prevented altogether and the swelling is often quite as extreme as in very dilute solutions of the acid alone. When still more salt is added, coagulation is postponed and there is no swelling, but instead a definite shrinkage which with Paramecium may amount to 50 per cent. With Euplotes it is difficult to measure the shrinkage, although it shows plainly in a collapse and curling

up of the edge of the cell. This shrinkage is probably a purely osmotic phenomenon, for it also occurs in a balanced salt solution such as diluted sea-water. The fat-soluble acids when acting alone produce a less well-marked coagulation and a greater disintegration; this is probably due in part to their power of dissolving lipoids and in part to the low concentration of the H ion present in the solutions. Otherwise the results are similar to those described for acetic.

It is especially noteworthy that in the efficiently balanced mixtures of salt and acid the protoplasm remains for a long time apparently normal, as if the acid were unable to penetrate the surface. The swelling which eventually appears in most of the mixtures is in all likelihood due to the small amount of acid which is able in spite of the salt protection gradually to penetrate the surface. Only in the presence of very small quantities of salt can enough acid penetrate to bring about coagulation. With more dilute acid solutions a dehydrating effect of the salts would probably be evident, and in the present experiments it may be indicated by the delay in the early development of the swelling.

DISCUSSION

There are in the literature a number of experiments illustrating the antagonism existing between acids and neutral salts, both in living and in non-living systems. Loeb² found that certain concentrations of acids (CO_2 , HCl , acetic, butyric) kill *Fundulus* adults apparently by altering the gill epithelium, and that the solutions are rendered almost harmless by the addition of NaCl or CaCl_2 . The effective concentration of NaCl is ten times that of CaCl_2 (in terms of molecular concentration). Similarly, the toxic action of isotonic sugar solutions upon *Fundulus* adults occurs only when the medium has become acid, and is greatly delayed by the presence of neutral salts (Loeb³). With *Fundulus* embryos Loeb⁴ found that the intake of water induced by acetic acid N/333 is greatly diminished by M/2 balanced salt solution. The toxicity of the acid to the embryo (as indicated by coagulation of the tail within the egg membrane) is also greatly lessened by the addition of neutral salts, probably because they hinder the diffu-

sion of the acid across the egg membrane by diminishing its state of hydration (Loeb^{5, 6}). It is also possible that at certain concentrations of acid the salts may stabilize the membrane by preventing coagulation, which if it occurred would destroy the continuity of the membrane and increase its permeability.

The normal electrical resistance of *Laminaria* (Osterhout⁷) is greatly lowered by HCl as well as by NaCl. But if the salt is added to the acid, the mixture, though still lowering the electrical resistance, produces a much more gradual effect than either the salt or the acid used alone. This agrees with the already known antitoxic action.

The kidney of the frog is normally impermeable to blood sugar but according to Hamburger and Brinckmann the degree of impermeability can be varied at will by altering either the salt content or the pH of the perfusion fluid. The ratio between Ca and the H ion seems to be of the utmost importance, although some differences are noticeable if the amount of K or Na is altered.

Lillie^{25, 26} has studied the relations of a rather more extensive series of salts and the capacity of NaCl and of HCl to diminish the toxicity of bivalent cations to the cilia of *Arenicola* larvae and of *Mytilus*. At the concentrations used NaCl induces swelling, while HCl and the chlorides of Ba, Sr and the heavy metals induce coagulation. Ca and Mg are intermediate, permitting some swelling, but gradually producing coagulation. In mixtures of NaCl with bivalent chlorides toxicity is much lessened, probably because the swelling influence of NaCl is balanced by the coagulative influence of Ba, etc. Clowes⁸ states that in solutions of NaCl the anion is taken up by most adsorbents more readily than the cation, while in solutions of CaCl₂ the cation is more strongly adsorbed. This would agree with Greeley's observation³⁷ that the protoplasm of *Paramecium* is liquefied by anions and coagulated by cations. So the antagonism would resolve itself into a balance between the adsorption and activity of the anion of NaCl and the cation of CaCl₂. Or it is possible that, as Loeb²³ suggests, the antagonism is between the cations and is due to differences in the solubility of the Na- and Ca-protein salts. Lillie also found that the toxicity of NaCl and to

some extent of CaCl_2 and MgCl_2 is reduced by traces of HCl , apparently because of approach to a balance between the coagulative action of the acid and the liquefying action of the salt. BaCl_2 and SrCl_2 are not susceptible of antagonism by the acid, perhaps because they coagulate the protoplasm readily at the concentration selected. In Lillie's experiments the concentration of salt (usually $\text{M}/2$) was much higher than in my experiments, which may account for the dissimilarity in the results, especially with Sr .

In some of the experiments cited the antagonism appears to be chiefly between the swelling action of dilute acid and the dehydrating effect of salts, while in others the antagonism is between the coagulative action of the concentrated acid (or salts with bivalent cation) and the liquefying or stabilizing action of a salt. That is to say, the type of antagonism varies with the concentration as well as with the nature of the antagonists.

This view is borne out not only by the occurrence of visible changes in the cell such as those already described, but also by experiments with non-living systems. With lecithin (Feinschmidt;¹¹ Handowsky and Wagner¹⁵) and also with globulin (Hardy¹⁴) the pH at which acid precipitation occurs is increased and the completeness of the precipitation is considerably limited by the presence of neutral salts. The acid swelling of gelatin (Fenn,¹² Fisher,¹³ Loeb,¹⁶ Proctor^{17, 18}), fibrin (Tolman and Stern²⁰) and gluten (Wood and Hardy²²) is diminished by salts, and a similar balanced is to be observed in the setting of gelatin (Traube and Kohler²¹). It is noteworthy that these substances are chemically unlike and agree only in being hydrophilous colloids.

That the acid acts largely at the surface of the cell is indicated by the experiments already cited (Collett, Loeb, Osterhout) on salt-acid mixtures and by many experiments on salt-salt and salt-anaesthetic mixtures. For example, the adsorption of Cl by tissue is less from a bath of NaCl plus CaCl_2 in physiological proportions than from a bath of either alone (van Oijen²⁰); the electrical resistance of *Laminaria* is decreased less by a balanced salt solution than by pure salts, and is increased less by a mixture of anaesthetic and NaCl than by anaesthetic alone

(Osterhout³²); the penetration of stimulating salts into a nerve is lessened by CaCl_2 , presumably by means of a surface action (Loeb and Ewald²⁷); the erosion of Na-glycocholate gels by NaCl is prevented by CaCl_2 and by anaesthetics (Schryver¹⁹). Lillie considers that the protective effect of Ca is due to its stabilizing action on the cell surface (as regards solution-coagulation balance) and in part to the greater solidity or insolubility of Ca-colloid compounds.

Höber,²⁴ in his extensive monograph on the physiological action of calcium, finds that Ca can be replaced by other alkaline earths (especially by Sr) better than by heavy metals, and still better by certain complex Co ions. In many cases, however, Ca is not wholly replaceable by any cation. Höber therefore concludes that the action is largely colloidal, but must also in part be chemical. Schreiter³³ notes that in the normal polarization of the nerve membrane,* Ca in Ringer's solution can be replaced by Ba and Sr, but not by substances which injure the membrane such as Mg, Mn, Co, Al. Mathews²⁰ would explain the difference in toxicity of various bivalent cations and in their power to antagonize NaCl, by the differences between them in solution tension, which would determine their ability to alter the surface energy relations of a colloid. The electromotive series runs: H Ni Co Cd Zn Mn Mg Ca Sr Ba Na. Here as in the order for toxicity the heavy metals are at one end of the scale and the alkaline earth metals at the other. This series, however, would not explain the great efficiency of Ca as compared with Sr and Ba nor the varying efficiency of Zn, Co, and Cd with different organisms. For that we must go back to specific chemical action or specific solubility for an explanation.

The antagonism of acid by salts may be due to protection of the membrane against penetration by the acid as a whole or to protection against the special action of the H ion. Thus the coagulative action of the H ion might be offset by the anticoagulative or dispersing action of the strongly adsorbed anion of such a salt as NaCl, or by competition with the strongly adsorbed cation

*I.e., alteration in distribution of dyes in axis cylinder when the nerve is stained during passage of a constant current along it.

of such a salt as CaCl_2 for possession of the cell surface. The liquefying action of more dilute acids on gelatin and similar gels might, as Loeb suggests, be limited by the ionization or specific solubility of the Na- or Ca-colloid salt. The penetration of the acid into the cell might be prevented by such changes in state at the surface as are suggested by Osterhout,³¹ Lillie,^{25,26} Spaeth,³⁴ and Fenn.¹² Sodium salts seem to promote the fluidity or degree of hydration and so the permeability of the membrane to water-borne substances, calcium salts to promote aggregation with dehydration of the surface layer and so impermeability (although Ca in excess will coagulate the membrane and so render it more permeable). Clowes^{8, 9, 10} has suggested that the increased aggregation of the film produced by CaCl_2 is due to an increase in the amount of water-insoluble soap in the most external boundary phase of the protoplasmic emulsion; such a change would be associated with lessened permeability to water-borne substances. The liquefaction induced by NaCl would be due to a decrease in the amount of the oil-miscible compound (lipoid or soap and an increase in the amount of the water-miscible compound (e.g., Na-soap); this change would lead to a greater permeability toward water-borne substances. These various explanations do not seem to be mutually exclusive although in some cases they do not apply equally well. Additive effects of various kinds must also enter.

In my experiments with *Paramecium*, Ca is a more efficient antagonist than NaCl and at a much lower concentration. This is true especially with the less fat-soluble acids. With *Euplotes* a similar difference is observed. At high concentrations NaCl completely loses its power to protect *Euplotes* against the non-fat-soluble acids, although at low concentrations it is fairly efficient. Against several fat-soluble substances NaCl proves to be an efficient antagonist, even at high concentrations, in spite of the fact that by itself it is very toxic to *Euplotes*. If we compare butyric with HCl in equitoxic concentrations, we find that NaCl at its best prolongs the life of *Paramecium* four times in butyric and three times in HCl; that of *Euplotes* ten times in butyric and once in HCl. That is, the efficiency of NaCl is

greater for both organisms with fat-soluble than with non-fat-soluble acids. And to some extent the reverse is true for Ca, though the difference is not marked.

	<i>HCl Butyric</i>			<i>Formic</i>	<i>Salicylic</i>
Paramecium:	3×	4×	NaCl	4×	7×
“	17×	17×	CaCl	12×	11×
Euplotes:	1×	10×	NaCl	2×	4×
“	20×	15×	CaCl	5×	5×

Perhaps we can explain these results by assuming that the action of each salt is double. Each has an influence upon phase reversal: pure NaCl solution decreases the proportion of water-insoluble lipoid and increases the water-soluble components (such as Na-soaps in the external phase of the protoplasmic emulsion, while CaCl_2 has the opposite effect. In addition each stabilizes the cell colloids against the swelling or coagulative action of the H ion, CaCl_2 better than NaCl. Sodium salts would diminish the ability of the fat-soluble acids to penetrate the cell by reducing the amount of lipoid or Ca-soap at the surface; consequently Na should protect against them better than against the non-fat-soluble acids which would be better able to penetrate a Na-soap. Calcium would increase the water-immiscible lipoid or soap and decrease the water-miscible soap at the surface, and so would render penetration more difficult to water-soluble acids and easier to fat-soluble acids, though this latter effect would be at least partially balanced by its power to stabilize the protoplasm against the action of the H ion. The efficiency of a salt would then depend upon the balance existing between its power to protect the membrane against the entrance of the acid molecule or anion, and its power to antagonize the H ion. Such a theory would be in accord with the behavior of acids of the two groups. Whether or not this explanation will hold in the form given here, it appears probable, judging from observations on visible changes in the state of the protoplasm, that the action, whatever its precise nature may be, takes place largely at the surface.

Nierenstein⁴¹ concludes from his work in vital staining that the protoplasm of Paramecium, not only at the surface, but also within

the cell-body, behaves like a lipoid containing traces of an organic acid and an organic base. In my experiments the phenomena of acid-induced hydration and coagulation suggest the presence of some protein at the surface of the cell, while the penetrating power of xylol and of such acids as butyric, caproic, benzoic, etc., indicates in addition a lipoid-like substance as an important component of the surface film. The facts observed in the antagonism of the acids by salts are consistent with Clowes' tentative identification of the surface lipoid as a soap, for the Na-soap would protect against lipoid-soluble substances and the Ca-soap against water-soluble substances. There may also be Na- or Ca-protein salts of unlike solubility, resembling the Na- and Ca-gelatin salts which Loeb¹⁰ has found to differ widely in hydration. My experiments, however, taken by themselves, are insufficient to establish the exact nature of the surface film or to explain the specific differences observed.

TABLE 7

HCl 0.00025 N	LENGTH OF LIFE IN MINUTES							
	Paramecium				Euplotes			
	0.0	0.01 0.001	0.05 1.005	0.1 0.01	0.0	0.01 0.001	0.05 0.005	0.1 0.01
Concentration of salts M. Monovalent cations..... Bivalent cations.....								
Alone.....	4				8			
Plus NaCl.....		8	12	9		8	6	6
Plus BaCl ₂		5	10	10		60	75	75
Plus CaCl ₂		30	70	65		110	120	180+
Plus MgCl ₂		5	7	7		12	12	11
Plus SrCl ₂		10	20	30		100	80	60
Plus BaCl ₂ plus NaCl....		11	20	15		120+	180	120+
Plus CaCl ₂ plus NaCl....		100	120	50		180+	180+	180+
Plus MgCl ₂ plus NaCl....		8	13	20		12	150	180+
Plus SrCl ₂ plus NaCl....		20	50	35		15	12	10
Plus CdCl ₂		1	1	1		12	10	10
Plus CoCl ₂		3	6	8		9	10	12
Plus MnCl ₂		7	12	18		11	15	30
Plus NiCl ₂		2				3		
Plus ZnCl ₂		2				9		

TABLE 8

FORMIC 0.0005 N	LENGTH OF LIFE IN MINUTES							
	Paramecium				Euplotes			
	0.0	0.01 0.001	0.05 0.005	0.1 0.01	0.0	0.01 0.001	0.05 0.005	0.1 0.01
Concentration of salts M. Monovalent cations..... Bivalent cations.....								
Alone.....	2½				6			
Plus NaCl.....		4	8	10		5	8	10
Plus Ba Cl ₂		3	4	5		12	10	10
Plus CaCl ₂		8	20	30		18	20	30
Plus MgCl ₂		3	4	5		6	7	6
Plus SrCl ₂		4	7	8		10	9	9
Plus BaCl ₂ plus NaCl....		4	6	5		9	35	40
Plus CaCl ₂ plus NaCl....		9	20	15		18	75	130
Plus MgCl ₂ plus NaCl....		4	6	5		6	15	10
Plus SrCl ₂ plus NaCl....		4	12	8		10	7	5

TABLE 9

PHENYL ACETIC 0.0005 N	LENGTH OF LIFE IN MINUTES							
	Paramecium				Euplotes			
	0.0	0.01 0.001	0.05 0.005	0.1 0.01	0.0	0.01 0.001	0.05 0.005	0.1 0.01
Concentration of salts M.								
Monovalent cations.....								
Bivalent cations.....								
Alone.....	2				2			
Plus NaCl.....		5	10	10		5	10	10
Plus KCl.....		2	4	5		2	4	5
Plus NH ₄ Cl.....		2	2	3		2	2	2
Plus BaCl ₂		3	4	5		15	22	30
Plus CaCl ₂		10	35	45		20	35	45
Plus MgCl ₂		3	4	4		3	3	4
Plus SrCl ₂		4	4	7		30	20	20
Plus BaCl ₂ plus NaCl....		4	7	6		25	120	45
Plus CaCl ₂ plus NaCl....		25	45	25		30	150	180
Plus MgCl ₂ Plus NaCl....		4	11	7		5	20	20
Plus SrCl ₂ plus NaCl....		6	15	10		25	120	110

TABLE 10

SALICYLIC 0.00015 N	LENGTH OF LIFE IN MINUTES							
	Paramecium				Euplotes			
	0.0	0.01 0.001	0.05 0.005	0.1 0.01	0.0	0.01 0.001	0.05 0.005	0.1 0.01
Concentration of salts M.								
Monovalent cations.....								
Bivalent cations.....								
Alone.....	2				5			
Plus NaCl.....		8	15	15		10	18	20
Plus KCl.....		5	8	7		10	11	18
Plus NH ₄ Cl.....		2	4	2		5	5	5
Plus BaCl ₂		4	6	5		8	13	12
Plus CaCl ₂		10	70	22		14	35	17
Plus MgCl ₂		4	5	7		5	10	7
Plus SrCl ₂		4	15	8		12	15	15
Plus BaCl ₂ plus NaCl....		5	13	10		12	14	10
Plus CaCl ₂ plus NaCl....		13	18	22		13	18	22
Plus MgCl ₂ plus NaCl....		7	15	7		7	15	10
Plus SrCl ₂ plus NaCl....		11	17	15		11	17	15

TABLE 11

LACTIC 0.0005 N	LENGTH OF LIFE IN MINUTES									
	Paramecium					Euplotes				
	0.0	0.005	0.01	0.05	0.1	0.0	0.005	0.01	0.05	0.1
Concentration of salts M.		0.0005	0.001	0.005	0.01		0.0005	0.001	0.005	0.01
Monovalent cations.....										
Bivalent cations.....										
Alone.....	2					8				
Plus NaCl.....			3	8	9			6	6	5
Plus KCl.....			3	4	4			6	7	8
Plus NH ₄ Cl.....			2	2	1			4	3	2
Plus BaCl ₂			4	7	12			30	50	30
Plus CaCl ₂			30	50	75			75	90	180+
Plus MgCl ₂			4	5	8			10	10	10
Plus SrCl ₂			6	15	30			45	65	40
Plus BaCl ₂ plus NaCl....			8	12	7			40	180	100
Plus BaCl ₂ plus NaCl....			45	80	17			90	180+	180+
Plus MgCl ₂ plus NaCl....			5	10	8			10	30	8
Plus SrCl ₂ plus NaCl....			12	14	12			25	8	7
Plus CdCl ₂			2	1	1			8	15	10
Plus CoCl ₂			2	4	3			6	8	8
Plus MnCl ₂			2	9	10			15	20	40
Plus NiCl ₂		2	<2	1			5	<5		
Plus ZnCl ₂		2	<2	<1			5	<5		

TABLE 12

TARTARIC 0.0005 N	LENGTH OF LIFE IN MINUTES							
	Paramecium				Euplotes			
	0.0	0.01	0.05	0.1	0.0	0.01	0.05	0.1
Concentration of salts M.		0.001	0.005	0.01		0.001	0.005	0.01
Monovalent cations.....								
Bivalent cations.....								
Alone.....	2				8			
Plus NaCl.....		4	8	8		8	4	4
Plus KCl.....		3	3	3		6	5	5
Plus BaCl ₂		3	7	8		25	40	40
Plus CaCl ₂		30	55	60		120	180+	150
Plus MgCl ₂		3	4	4		8	9	9
Plus SrCl ₂		8	15	15		25	80	80
Plus BaCl ₂ plus NaCl....		5	9	5		45	180+	100
Plus CaCl ₂ plus NaCl....		25	45	25		180	180+	180+
Plus MgCl ₂ plus NaCl....		5	10	10		12	180	180+
Plus SrCl ₂ plus NaCl....		8	15	15		30	40	10

TABLE 13

CITRIC 0.0005 N	LENGTH OF LIFE IN MINUTES									
	Paramecium					Euplates				
	0.0	0.005 0.0005	0.01 0.001	0.05 0.005	0.1 0.01	0.0	0.005 0.0005	0.01 0.001	0.05 0.005	0.1 0.01
Concentration of salts M.										
Monovalent cations.....	0.0					0.0				
Bivalent cations.....										
Alone.....	2					10				
Plus NaCl.....			7	15	11			8	11	10
Plus KCl.....			3	5	4			10	8	10
Plus NH ₄ Cl.....			3	3	3			4	3	2
Plus BaCl ₂			6	10	10			80	15	20
Plus CaCl ₂			40	90	85			180+	140	140
Plus MgCl ₂			6	7	7			9	10	15
Plus SrCl ₂			10	20	30			80	70	60
Plus BaCl ₂ plus NaCl.....			5	10	5			90	20	8
Plus CaCl ₂ plus NaCl.....			30	100	40			180	120+	180
Plus MgCl ₂ plus NaCl.....			10	12	8			10	50	18
Plus SrCl ₂ plus NaCl.....			12	20	18			35	20	7
Plus CdCl ₂			1	1	1			15	10	15
Plus CoCl ₂			2	3	3			5	7	8
Plus MnCl ₂			4	9	12			30	18	20
Plus NiCl ₂		2	<2				5	4		
Plus ZnCl ₂		<2	<2				5	6		

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Resumen por los autores Raymond Pearl y William F. Schoppe.

Estudios sobre la fisiología y reproducción de la gallina doméstica. XVIII. Nuevas observaciones sobre la base anatómica de la fecundidad.

Como continuación de trabajos precedentes, los autores dan a conocer en el presente los resultados del recuento de los ovocitos visibles en los ovarios de 36 aves, que comprenden varias razas de gallinas domésticas, gallinas acuáticas y aves salvajes. En general, el número medio de ovocitos visibles en los ovarios de las diferentes clases de aves refleja la fecundidad normal o actividad de la puesta de las mismas clases de aves. Esta relación no representa con exactitud o regularidad las diferencias entre la fecundidad de los diversos individuos. En las gallinas de la variedad atigrada de la raza Plymouth Rock el número de ovocitos visibles aumenta con la edad del individuo, entre los límites de seis meses a treinta y siete meses. La probable explicación de este fenómeno es objeto de exposición por los autores. Extirpando una porción del ovario y provocando su regeneración, el número total de ovocitos que se desarrollan hasta alcanzar tamaño visible durante la vida del ave aumenta desde 33 hasta 68 por ciento sobre el número que se desarrolla en una gallina normal, no operada. Los autores demuestran que este aumento es de importancia bajo el punto de vista estadístico.

Translation by José F. Nonidez
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STUDIES ON THE PHYSIOLOGY OF REPRODUCTION IN THE DOMESTIC FOWL

XVIII. FURTHER OBSERVATIONS ON THE ANATOMICAL BASIS OF FECUNDITY¹

RAYMOND PEARL AND WILLIAM FREEMAN SCHOPPE

TWO FIGURES

INTRODUCTION

Some eight years ago one of us² published the results of some counts of the visible oöcytes upon the ovaries of domestic fowls. The number of birds dealt with at that time was statistically small, amounting to but seventeen. The problem to which attention was directed in this earlier work was stated as follows:

"To what extent are observed variations in fecundity (i.e., in the number of eggs laid) to be referred to anatomical differences? In other words, does the ovary of a high-producing hen, with, for example, a winter record of from 75 to 115 eggs, contain a larger number of oöcytes than does the ovary of a hen which is a poor producer, laying no eggs in the winter period and perhaps but 10 or 15 eggs in the year?"

The purposes of the present work were to extend the observations to a larger number of birds; to check the general reliability of the results by getting independent counts of another observer; to get data on wild birds, and to carry out certain experiments on ovarian regeneration. The counts of the present series will be combined with the earlier data and the whole submitted to certain biometrical treatment.

¹Papers from the Department of Biometry and Vital Statistics, School of Hygiene and Public Health, Johns Hopkins University, no. 24.

The observations on which this paper is based were made some years ago at the Maine Agricultural Experiment Station. The paper has been completed by the senior author.

²Pearl, R. The mode of inheritance of fecundity in the domestic fowl. *Jour. Exp. Zool.*, vol. 13, pp. 153-268, 1912.

In dealing, in this and the earlier work referred to, with oöcytes visible to the unaided eye alone, the tacit assumption is that the number of such visible ova is correlated to a high degree with the undetermined—and indeed undeterminable—total number of ova in the ovary. The work of von Hanseman³ and of Käppeli⁴ indicates that this assumption is justified. Occasionally a case turns up, as will be shown presently in detail, where the physical condition of the bird's ovary as a whole greatly raises the limit of oöcyte visibility, so to speak. This condition, however, we have found to occur but once in nearly forty birds examined from this point of view. So far as concerns the remainder of the counts, we are convinced, after direct experience with the material, that the ratio between visible ova and mass of the ovary as a whole, and by inference the number of invisible ova, is normally a rather constant one, and that consequently our material is as homogeneous and trustworthy as any statistical data on the soft parts of animals.

Regarding the method of counting, the following may be added to what was said in the earlier paper (*loc. cit.*, p. 296).

The ovaries, when taken from the birds, were placed in a 10 per cent solution of formalin, in order to preserve them until counts could be made. In making the earlier counts, fresh ovaries were at first used, but later after trying various preservatives it was concluded that the use of formalin was most satisfactory, as it preserved the ovaries in perfect condition and made counting possible at any time.

The functioning ovary when taken from the bird closely resembles a bunch of grapes. It is made up of a large number of oöcytes held together by connective tissue, joined to a heavy stalk by which they are held in place in the body.

The oöcytes are of various sizes, and for convenience in counting four divisions were made: *a*) follicles from which the ova have been discharged; *b*) oöcytes over 1 cm. in diameter; *c*) those over 1 mm. and less than 1 cm.; *d*) those less than 1 mm.

³Hansemann, D. Ueber den Kampf der Eier in den Ovarien. *Arch. f. Entwicklungsmech.*, 1912, Bd. 35, S. 223-235, 1 pl.

⁴Käppeli, I. Beiträge zur Anatomie und Physiologie der Ovarien von wildlebenden und gezähmten Wiederkäuern und Schweinen. Bern, 1908, K. J. Wyss. 78 pp., 5 tab., 6 pl.

Since the first class indicates the number of discharged ova, it theoretically ought to conform closely with the total number of eggs laid. However, as the follicles are being constantly absorbed they eventually reach a stage where it is difficult to determine positively if they are follicles or parts of connective tissue. It was practically impossible to measure every oöcyte, consequently the last three divisions are approximate, but conform very closely, since, if there was any doubt, the oöcyte was measured.

In making the counts small pieces of the ovary were separated, placed in a dish of water, and teased apart in order to distinguish the ova. Only such ova were counted as could be seen with the unaided eye. A low-power dissecting lens was used in this work, not to find more ova, but to assist in more readily and accurately counting those that could be seen.

DATA

In table 1 are collected together all the data collected at the Maine Station on the number of visible oöcytes in the ovaries of birds, including the counts of the earlier paper as well as those of the present investigation. The old counts are designated by asterisks. Within each breed the birds are arranged in descending order of total number of visible oöcytes.

A word should be said concerning cases 25, 26, 27 and 28. The two Pekin ducks were both in a healthy condition and ovaries were apparently functioning. In the case of no. 26 this was certain, since an egg was found in the uterus. In case of no. 28, a Toulouse goose, the ovary seemed to be in a very unhealthy condition, as all the larger and some of the smaller oöcytes were being absorbed. The tissue was very tender and several oöcytes were broken in removing the ovary from the body and in placing it in the preservative, so that although the ovary is credited with two follicles it is doubtful if the bird ever laid an egg.

Case no. 27, a young goose, hatched in the spring of 1912, proved a very desirable bird. She had a large ovary (199 grams) in a very active stage of production. An egg was found in the oviduct. The eleven oöcytes over 1 cm. in diameter ranged in weight from 2 to 32 grams. The five largest weighed 32, 31.5,

TABLE I
Showing the number of visible oöcytes in the ovary of certain birds

CASE NUMBER	BREED OR SPECIES	DATE HATCHED	DATE KILLED	TOTAL EGGS LAID	WINTER PRODUCTION	DISCHARGED FOLLICLES	OÖCYTES OVER 1 CM. IN DIAMETER	OÖCYTES 1 MM. TO 1 CM. IN DIAMETER	OÖCYTES UNDER 1 MM. IN DIAMETER	TOTAL VISIBLE OÖCYTES
A. Domestic fowl (<i>Gallus</i> sp.)										
*1	Barred Plymouth Rock	Apr. 28, '10	Apr. 4, '11	34	3	49	7	29	2121	2306
2	Barred Plymouth Rock	Spring, '07	May, 14, '10	412	82	31	7	70	2191	2299
*3	Barred Plymouth Rock	June 2, '10	Mar. 24, '11	15	0	17	6	49	2029	2101
4	Barred Plymouth Rock	May 11, '10	Nov. 2, '12	205	43	99	2	136	1775	2102
*5	Barred Plymouth Rock	June 2, '10	Mar. 30, '11	10	0	12	7	51	1596	1666
6	Barred Plymouth Rock	June 1, '10	Nov. 4, '12	63	4	74	0	297	1275	1646
*7	Barred Plymouth Rock	May 19, '10	Mar. 17, '11	19	5	24	5	92	1455	1576
*8	Barred Plymouth Rock	Mar. 30, '09	July 7, '10	23	0	21	12 ¹	142	1346	1521
9	Barred Plymouth Rock	Mar. 28, '11	Sept. 29, '11	5		18	8	57	1274	1357
*10	Barred Plymouth Rock	June 1, '10	Mar. 28, '11	10	3	17	9	53	1149	1228
*11	Barred Plymouth Rock	June 2, '10	Mar. 24, '11	16	0	23	6	42	1123	1194
*12	Barred Plymouth Rock	June 2, '10	Mar. 14, '11	17	5	12	8	68	1096	1174
*13	Barred Plymouth Rock	June 1, '10	Mar. 10, '11	7	0	8	5	62	839	914
*14	White Leghorn	May 28, '09	Dec. 15, '10	197	32	217	1	108	3279	3605
*15	White Leghorn	May 18, '09	Dec. 20, '10	198	54	75	2	231	2146	2452
*16	White Leghorn	June 14, '09	Dec. 22, '10	2	0	43	2	80	2022	2145
*17	White Leghorn	May 21, '09	Dec. 13, '10	10	0	11	3	75	1626	1701
*18	Cornish Indian Game	Apr. 21, '09	July 12, '10	52	13	54	6	167	1323	1550
19	Light Brahma	?	May 25, '12	?	?	28	9	119	1074	1230
20	F, Cross-bred	May 2, '11	Apr. 9, '12	46	30	47	6	111	2622	2786

21	F ₁ Cross-bred	Apr. 26, '11	June 14, '12	143	73	52	18	52	2418	2540
22	F ₁ Cross-bred	Apr. 21, '11	June 14, '12	91	38	42	5	43	2239	2529
23	F ₁ Cross-bred	Mar. 29, '11	June 26, '12	48	17	40	7	46	2128	2221
*24	F ₁ Cross-bred	Mar. 31, '10	Mar. 20, '11	124	106	50	5	70	1875	2000

B. Other domestic birds

25	Pekin duck	?	June 12, '12	?	?	10	19	276	892	1197
26	Pekin duck	Spring, '12	Mar. 27, '13	?	?	16	5	141	777	939
27	Toulouse goose	Spring, '12	Apr. 9, '13	?	?	12	11	129	1770	1922
28	Toulouse goose	Spring, '12	Apr. 8, '13	?	?	2	18	55	982	1057
*29	Guinea-hen	?	Jan. '11	?	?	9	3	36	717	765
*30	Guinea-hen	?	Jan. '11	?	?	0	3	38	545	586

C. Wild birds

31	Crow (<i>Corvus brachyrhynchos</i>)	?	July 10, '12	?	?	0	0	0	808	808
32	Crow (<i>Corvus brachyrhynchos</i>)	?	Apr. 11, '12	?	?	0	0	13	708	721
33	Kingbird (<i>Tyrannus tyrannus</i>)	?	Apr. 27, '12	?	?	0	0	0	505	505
34	Kingbird (<i>Tyrannus tyrannus</i>)	?	Apr. 28, '12	?	?	0	0	0	247	247
35	Meadow-hawk	?	Apr. 8, '12	?	?	0	0	0	102 ³ 135 ⁴	237

¹ This includes eight yolks in process of absorption.² Bird not in laying condition when killed.³ Right ovary.⁴ Left ovary.

27, 23.5 and 21 grams. The last six weighed 14, 12.7, 12, 8, 3.5, and 2 grams, and seemed to constitute a series which coincided closely with weights taken of the larger oöcytes in hens.

Theoretically, the number of discharged follicles and the total number of eggs laid should agree. Actually, this condition is never absolutely realized in the counts. In some cases it will be found that the count of follicles is greater than the total eggs laid. This can be accounted for in four ways: *a*) the bird may have laid while still on the range or before she was put in the laying house; *b*) eggs may have been laid on the floor before the birds became accustomed to using the nests; *c*) some of the yolks may have missed the oviduct, being deposited in the body cavity, and eventually absorbed; *d*) in some cases pieces of projecting connective tissue may have been mistaken for a follicle in making the counts. In other cases it will be seen that the eggs laid exceeded the number of follicles found. This is directly due to not being able to distinguish the follicles after they had become nearly or completely absorbed.

In addition to the thirty-five counts listed in table 1, the oöcytes of one other F_1 cross-bred bird were counted on a somewhat different plan. The ovary presented an extraordinary discreteness of separation of even minute oöcytes. In this case a low-power dissecting lens was used in the counting, and all oöcytes were enumerated which could be counted with the aid of the lens. The results were as follows:

Case No. 36

Lens aided count

F_1 crossbred

Hatched April 21, 1911

Killed June 14, 1912

Total eggs laid = 69

Winter production = 46

Discharged follicles = 29

Oöcytes > 1 cm. = 2

Oöcytes 1 mm. to 1 cm. = 80

Oöcytes < 1 mm. = 13,365

Total oöcytes = 13,476

This case shows the enormous difference which the physical construction of the ovary may make in the number of visible oöcytes in exceptional cases. Since this condition has been seen but this once in many hundreds of fowl ovaries examined, it cannot be considered in any way to invalidate the counts of normal ovaries (in the sense of visibility of oöcytes) given in table 1.

With the data before us, we may proceed to their biometrical analysis along certain lines.

TABLE 2
Constants deduced from table 1

BREED OR SPECIES	MEAN NUMBER OF VISIBLE OÖCYTES	STANDARD DEVIATION	COEFFICIENT OF VARIATION
White Leghorn fowls.....	2476	705	28.5
F ₁ Cross-bred fowls.....	2415	274	11.4
Barred Plymouth Rock fowls.....	1615	433	26.8
All fowls counted.....	1906	615	32.3
Toulouse geese.....	1489	432	29.0
Pekin ducks.....	1068	129	12.1
All domestic water-fowl counted.....	1279	382	29.9
Guinea-hens.....	676	90	13.3
Crows.....	764	44	5.8
Kingbirds.....	376	129	34.3
All wild birds counted.....	504	235	46.7
All wild birds and guinea-hens.....	553	219	39.6

BREED AND SPECIES COMPARISONS

The first point for consideration is the relation of the number of visible oöcytes to laying capacity in the different breeds. It is evident enough from simple inspection of table 1 that there is no correlation among individual fowls between visible oöcytes and egg production. The case may, however, be different when the comparison is made between breeds. As a necessary preliminary to the discussion of this point, table 2 is presented, containing the required biometric constants.

It is evident from table 2 that, considering broad categories, the mean number of visible oöcytes falls in the same order as the normal egg production of the kind of bird concerned. The domestic fowls stand at the head of the birds considered in respect of fecundity and also in number of visible oöcytes. The wild birds (including the guinea-hens) stand at the bottom of the list in both respects, while the domestic water-fowl occupy an intermediate position in both respects. The guinea-fowls are included in the average with wild birds for the reason that in its reproductive activities this creature behaves in all essentials like a wild bird. It refuses to lay except in a hidden nest of its own choosing, and lays in the course of a year a single clutch of not more than fourteen to sixteen eggs.⁵

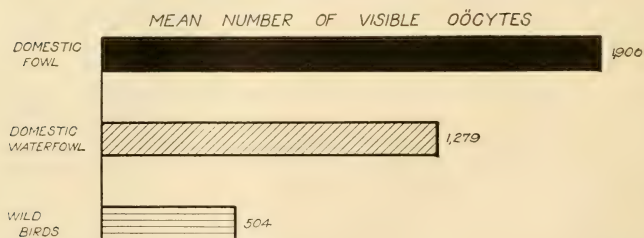


Fig. 1 Showing average number of visible oöcytes on ovary of, a) domestic fowl, b) domestic waterfowl, and, c) wild birds.

The relations of the three group averages discussed are shown graphically in figure 1.

It is of interest to note the considerably higher average number of visible oöcytes in the F_1 cross-breeds than in the pure Barred Plymouth Rocks. All of these cross-breeds were genetically half Barred Plymouth Rock, of the same strain as the pure-bred birds furnishing the counts and half from some other breed of lower fecundity than the Barred Rocks. The fact that the F_1 birds show a relatively high ovarian count, roughly 20 per cent higher than the average of the more fecund parent strain,

⁵These statements apply, from personal observation, certainly to the strain of guinea-fowls from which the birds here counted came.

suggests that we may have here a result due to heterosis, of the same sort essentially as those which have been observed particularly by East⁶ in plants.

The coefficients of variation for these ovarian counts fall, in the longer and hence more reliable series, in the neighborhood of a value of 30 per cent. This is about the value found for the relative variability of a number of physiological characters, particularly fecundity of the domestic fowl as measured by annual egg production.⁷

RELATION OF OÖCYTE NUMBER TO AGE

It is a well-known fact that in the domestic fowl egg production rapidly diminishes after the first year or in some breeds such as the Leghorns the first two years of life. Fowls may live for a considerable number of years, but generally after the early years they lay no eggs at all, or at most one small clutch in the spring.

In the case of Barred Plymouth Rocks, of the Maine Station strain, egg production usually practically ceases after the bird is two years old. It is commonly assumed that this cessation or great reduction in fecundity with age is due to atrophic processes in the ovary. It is, on this account, of interest to see in what manner the counts of visible oöcytes change with age.

Unfortunately, the material in which the point can be tested is meager. The Barred Plymouth Rocks constitute the only group sufficiently large, and there is a highly uneven distribution by age. Of the thirteen birds, nine were under one year of age when killed, one was between one and two, two between two and three, and one between three and four. It will be worth while, however, to see what this admittedly meager material shows. The data are exhibited in table 3 and graphically in figure 2.

In so far as the data may be trusted as fairly representative of what generally occurs, they indicate that the number of visible oöcytes increases with age, within the age limits of the observa-

⁶Cf. East, E. M., and Jones, D. F. Inbreeding and outbreeding. 1919.

⁷Cf. Pearl, R., and Surface, F. M. A biometrical study in egg production in the domestic fowl. I. Variation in annual egg production. U. S. Dept. of Agric., Bur. of An. Ind. Bull. 110, Part I.

tions. Apparently what happens in the ovary of the fowl after the bird reaches laying age is that ova are constantly migrating to the surface and developing thereon up to something under 1 mm. in diameter. Of these a much smaller number continue

TABLE 3

Mean number of visible oöcytes in Barred Plymouth Rocks of different ages

AGE CLASS IN YEARS	MEAN AGE OF GROUP IN MONTHS	NUMBER OF BIRDS	MEAN NUMBER OF VISIBLE OÖCYTES
Under 1	9.48	9	1502
1 to 1.9	15	1	1521
2 to 2.9	29.25	2	1829
3 to 3.9	37	1	2299

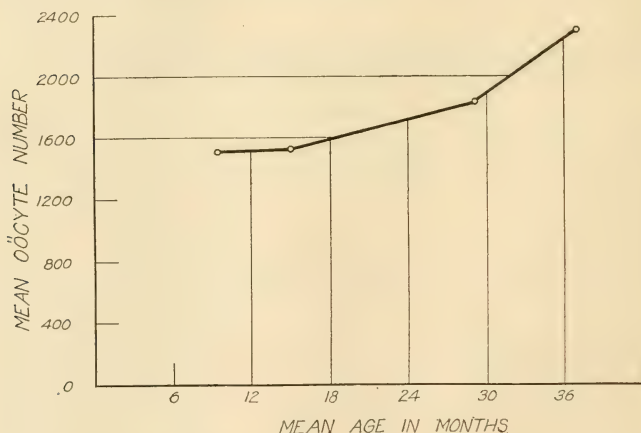


Fig. 2 Showing the change in mean number of visible oöcytes with advancing age in Barred Plymouth Rocks.

their growth and are finally laid. Since the number developing up to the 1-mm. size, however, always greatly exceeds in any given time unit the number developing farther to laying size, and since this disproportion may be reasonably assumed to become progressively greater as laying activity diminishes, which it is

known to do with advancing age, and since, as table 1 shows, the small oöcytes under 1 mm. in diameter make up the vast majority of all those visible, it follows that we should expect to find more and more visible oöcytes as age advances up to an asymptote which represents the complete cessation of all ovarian activity. Presumably, the oldest of the birds here counted was not old enough to have reached this condition of complete ovarian rest. Our general knowledge of ovarian functioning in fowls, from personal observation, would lead to this last conclusion as well as the form of the curve in figure 2.

Probably the complete curve for visible oöcytes on an age base, if we had data to plot it, would be of the shape of a flattened S, with an upper asymptote, representing complete ovarian rest, and a lower zero asymptote representing no visible oöcytes at an early age. This is a form of curve which has been found to represent growth phenomena of various sorts.⁸

EXPERIMENTS ON OVARIAN REGENERATION

It is well known that the ovary in birds is an organ that regenerates readily and freely after injury. Indeed, this fact constitutes one of the chief technical difficulties in making a complete and permanent castration of female birds. At the same time it has been held as a basic biological doctrine that during the life of the individual there neither is nor can be any increase in the number of primary oöcytes beyond those originally laid down when the ovary is formed. It occurred to us to make a quantitative study of visible oöcytes in regenerated fowls' ovaries to determine to what extent an increase could be brought about in the number of primary oöcytes which develop to visible size at the

⁸Robertson, T. Brailsford. On the normal rate of growth of an individual and its biochemical significance. *Arch. für Entwicklungsmechanik der Organismen*, Bd. 25, S. 581-514.

Robertson, T. Brailsford. Further remarks on the normal rate of growth of an individual, and its biochemical significance. *Arch. für Entwicklungsmechanik der Organismen*, Bd. 26, S. 108-118.

Pearl, R., and Reed, Lowell J. On the rate of growth of the population of the United States since 1790 and its mathematical representation. *Proc. Nat. Acad. Sci.*, vol. 6, no. 6, 1920.

surface of the ovary. Other experiments had indicated that by inducing regeneration of the ovary a bird could be caused to lay more eggs than she would have had there been no interference with the ovary. The present experiments were undertaken as a quantitative test of this suggested result.

Eight birds were used in the experiments, four Barred Plymouth Rocks and four Cornish Indian Games. The operation protocols follow:

Bird no. 9017, operation 131. Bird in find condition, large yolks being absorbed; about five-sixths of the ovary removed with almost no loss of blood.

Bird no. 9030, operation 129. Bird not in laying condition. Largest yolk about 8 mm. in diameter. Ovary in good healthy condition; about two-thirds was removed.

Bird no. 9026, operation 128. Bird in laying condition. large yolks present; one-half of ovary removed. There was considerable bleeding.

Bird no. 9022, operation 127. Bird in nearly laying condition but had stopped temporarily. Large yolks present; one-half of ovary removed. There was considerable bleeding.

Bird no. 9018, operation 125. Bird apparently healthy. Ovary in very bad condition. Large number of absorbing yolks and yolk tumors. Evidently septic condition, as all peritoneal surface was thick and opaque. Removed large part of ovary and all tumors. There was considerable bleeding.

Bird no. 9023, operation 123. Bird in healthy condition. Ovary not in laying condition; about two-thirds removed.

Bird no. 9019, operation 126. Bird in healthy condition. Ovary healthy, but in a non-laying condition. Less of ovary removed than in either 127, 125, or 123.

The pieces of removed ovary were placed in formalin and counts later made.

It was the original intention to keep these birds until they had all started to lay, but this was found impossible, owing to lack of time. As it was, half of the birds had started to lay, and it was only a question of time before the others would begin, as all the ovaries were in a very healthy condition.

Bird no. 9017 was the only one that died. An autopsy showed that the ovary was in an apparently normal condition. There were three absorbing yolks. The oviduct was normal, and as the bird had been laying her death could not be attributed to the operation.

Bird no. 9030 was killed to secure the ovary. The autopsy showed her to be in a very healthy condition. There were four large normal yolks in the ovary and one absorbing yolk. There was also an egg in the oviduct.

Bird no. 9026, killed to secure ovary. She was in a normal healthy condition. There were three large yolks in the ovary and an egg in the oviduct. Ovary of fair size, but not as large as in a normal bird.

Although bird no. 9022 had not laid, she was killed. It was found that the ovary was in a laying condition, containing five large yolks. Several yolks were found in the body cavity surrounded by adhesions. There was no apparent reason for this, as the oviduct seemed to be normal. The funnel was in its normal position and was not injured.

Bird no. 9018. It will be recalled that at the time of the operation this bird had a very bad ovary, containing several absorbing yolks and yolk tumors. When killed, although she had not laid, it was found that the ovary had several enlarging yolks and that the oviduct was enlarging. There were no tumors or septic tissue. Evidently the operation had a beneficial effect.

Bird no. 9023 was the only one whose ovary showed no apparent growth. Both the ovary and oviduct were in a healthy condition, but small.

The two days preceding the day on which bird no. 9019 was killed she layed. Her ovary was found to contain two large normal yolks and one absorbing yolk.

Bird no. 9025 did not lay; when killed the ovary was found to be healthy. It was enlarging, as was also the oviduct.

Counts were made in the usual way of *a*) visible oöcytes on the portion of ovary removed at the original operation and, *b*) visible oöcytes on the regenerated ovary at the death of the bird.

The results of these counts together with other pertinent data are given in tables 4 and 5.

The data of tables 4 and 5 are brought together for comparison in table 6, which gives in sum the total oöcytes counted.

TABLE 4

Showing number of visible oöcytes in part of ovary removed from birds by operation

BIRD NUMBER	BREED	DATE OF HATCHING	DATE OF OPERATION	TOTAL EGGS LAID BEFORE OPERATION	DISCHARGED FOLLICLES	OÖCYTES OVER 1 CM.	OÖCYTES BETWEEN 1 MM. AND 1 CM.	OÖCYTES UNDER 1 MM.	TOTAL VISIBLE OÖCYTES
9017	B. P. R.	3/30/11	9/12/12	124	26	7	119	854	1006
9030	B. P. R.	3/10/11	9/12/12	125	7	1	75	238	321
9026	B. P. R.	4/ 1/11	9/12/12	125	29	4	67	623	723
9022	B. P. R.	3/29/10	9/12/12	79	17	1	109	476	603
9018	C. I. G.	5/19/10	9/10/12	42	14	7	97	1131	1249
9023	C. I. G.	6/ 2/10	9/10/12	35	16	0	77	701	794
9019	C. I. G.	3/31/10	9/10/12	26	9	0	102	1265	1376
9025	C. I. G.	4/ 1/10	9/10/12	31	12	0	140	780	932

TABLE 5

Showing number of visible oöcytes in ovary of operated birds when killed

BIRD NUMBER	BREED	DATE OF HATCHING	DATE OF KILLING	DATE OF OPERATION	FIRST EGG LAID AFTER OPERATION	TOTAL EGGS LAID AFTER OPERATION	DISCHARGED FOLLICLES	DIAMETER OF OÖCYTES			TOTAL VISIBLE OÖCYTES
								Over 1 cm.	1 cm. to 1 mm.	Under 1 mm.	
9017	B. P. R.	3/30/11	2/24/13	9/12/12	2/17/13	13	10	3	94	1874	1981
9030	B. P. R.	5/10/11	3/31/13	9/12/12	1/11/13	25	9	5	122	1197	1333
9026	B. P. R.	4/ 1/11	3/31/13	9/12/12	3/27/13	2	11	5	100	1435	1551
9022	B. P. R.	3/29/10	4/ 2/13	9/12/12		0	1	7	98	977	1083
9018	C. I. G.	4/19/10	4/ 3/13	9/10/12		0	0	2	92	936	1030
9023	C. I. G.	6/ 2/10	4/ 3/13	9/10/12		0	0	0	73	1125	1198
9019	C. I. G.	3/31/10	4/ 3/13	9/10/12	4/ 1/13	2	7	3	120	1977	2107
9025	C. I. G.	4/ 1/10	4/ 3/13	9/10/12		0	2	1	177	1578	1758

From table 6 it is seen that in all cases except one (bird no. 9018) the number of visible oöcytes on the piece removed was smaller than the number found on the regenerated ovary at

TABLE 6

Showing total number of visible oöcytes in ovary of operated birds

BIRD NUM- BER	BREED	DATE OF HATCH- ING	DATE OF KILLING	DATE OF OPERA- TION	FIRST EGG LAID AFTER OPERA- TION	TOTAL NUM- BER LAID IN LIFE	DIS- CHARG- ED FOLLI- CLES	DIAMETER OF OÖCYTES			TOTAL VISIBLE OÖCYTES
								Over 1 cm.	1 cm. to 1 mm.	Under 1 mm.	
9017	B. P. R.	3/30/11	3/24/13	9/12/12	2/17/13	124	26	7	119	854	1006
						13	10	3	94	1874	1981
						Total	137	36	10	213	2728
9030	B. P. R.	5/10/11	3/31/13	9/12/12	1/11/13	125	7	1	75	238	321
						25	9	5	122	1197	1333
						Total	150	16	6	197	1435
9026	B. P. R.	4/1/11	3/31/13	9/12/12	3/27/13	125	29	4	67	623	723
						2	11	5	100	1435	1551
						Total	127	40	9	167	2058
9022	B. P. R.	3/29/10	4/2/13	9/12/12		79	17	1	109	476	603
							1	7	98	977	1083
						Total	79	18	8	207	1453
9018	C. I. G.	5/19/10	4/3/13	9/10/12		42	14	7	97	1131	1249
								2	92	936	1030
						Total	42	14	9	189	2067
9023	C. I. G.	6/2/10	4/3/13	9/10/12		35	16		77	701	794
									73	1125	1198
						Total	35	16		150	1826
9019	C. I. G.	3/3/10	4/3/13	9/10/12	4/1/13	26	9		102	1265	1376
						2	7	3	120	1977	2107
						Total	28	16	3	222	3242
9025	C. I. G.	4/1/10	4/3/13	9/10/12		31	12		140	780	932
							2	1	177	1578	1758
						Total	31	14	1	317	2358

death, as would be expected. The operation protocol on bird no. 9018 shows that the ovary was in a diseased condition at the time of operation, but at the time of killing the ovary was in normal condition. The protocol states that a "large part of ovary was removed." So far as could be estimated, a larger portion of the ovary was removed at operation in this case than in any other, with the possible exception of 9017.

In order to make clearer comparisons, table 7 has been calculated from the data of table 6.

TABLE 7
Constants from regeneration experiments

GROUP	TOTAL VISIBLE OÖCYTES		
	Mean	Standard deviation	Coefficient of variation
Portion removed—Barred Plymouth Rocks	663 ± 83	246	37.1
Regenerated ovary—Barred Plymouth Rocks . . .	1487 ± 110	328	22.1
Total—Barred Plymouth Rocks	2150 ± 183	543	25.2
Normal Barred Plymouth Rocks (ex. table 2) . .	1615 ± 81	433	26.8
Portion removed—Cornish Indian Game	1088 ± 79	234	21.5
Regenerated ovary—Cornish Indian Game	1523 ± 146	432	28.3
Total	2611 ± 189	561	21.5
Normal Cornish Indian Game (ex. table 2)	1550 ¹		

¹ One case only.

From these data a number of points of interest appear. In the first place, it is evident that the number of visible oöcytes on the regenerated ovaries is substantially the same as the mean number on the normal unoperated ovary of the same breed. In the case of the Barred Rocks the difference is $1615 - 1487 = 128$, an obviously insignificant difference. In the Cornish Indian Games the difference is even smaller, amounting to $1550 - 1523 = 27$. In the latter case we have, unfortunately, only a single normal count, rather than a normal mean.

If we add to the number of oöcytes found upon the regenerated ovary those upon the piece removed at operation to get the total number of visible oöcytes the bird produced in its life, we get a considerable excess over the number produced by normal un-

operated ovaries of the same bird. This excess amounts in the case of the Barred Rocks to $2150 - 1615 = 535$, an increase of 33 per cent in operated over normal, and in the case of the Cornish Indian Games to $2611 - 1550 = 1061$, an increase of 68 per cent in operated over normal. The excess shown by the Barred Rocks can hardly be regarded as statistically significant, on the basis of the present short series. The fact, however, that both breeds show such a consistent set of results throughout is evidence that with a longer Barred Rock series we should get essentially the same result as with the present one and with sufficiently reduced probable errors to make the difference statistically significant. A fair and instructive comparison can be made by lumping together the Barred Rock and Game operated series, and putting the mean total number of visible oöcytes produced during life against the mean total number produced by all normal unoperated fowl, excluding the White Leghorns, since our previous results show that this breed has evidently as a breed character a distinctly larger number of visible oöcytes than any of the other breeds dealt with. On this basis we have:

Mean total visible oöcytes produced during life by eight operated birds.....	2381±143
Mean total visible oöcytes produced during life by twenty normal birds.....	1793± 79
Difference.....	588±163

Here we see that the difference is 3.6 times its probable error, and hence to be regarded as almost certainly statistically significant.

Altogether it is believed that we may safely conclude that by inducing by operative means ovarian regeneration in the domestic fowl the total number of primary oöcytes which develop to visible size is significantly increased.

Following these experiments the senior author undertook much more extensive work on repeated ovarian regeneration. In a series of birds of known and highly interesting genetic constitution (F_2 birds of a carefully studied cross) a considerable portion of the ovary was removed each autumn for four years. The laying record was kept over this long period, and also careful

record made of the chicks which developed from oöcytes produced by these regenerated ovaries. Unfortunately, all the records of this experiment were lately destroyed by fire, and hence cannot be reported. The writer regarded this experiment as perhaps the most interesting and fundamentally significant for the problem of genetics of any of his work, with poultry. Mention is made of the matter here in the thought that possibly some one so situated as to be able to carry on work of this sort, which the writer no longer is, may care to repeat the work.

SUMMARY

The chief results of this work may be stated as follows:

1. Detailed counts are given of the number of visible oöcytes on the ovaries of thirty-six birds, including various breeds of domestic fowls, water-fowl, and wild birds.

2. In general the mean number of visible oöcytes on the ovaries of different kinds of birds reflects the normal fecundity or laying activity of the same kinds of birds. This relation does not hold with any exactitude or regularity for differences in fecundity between individual birds.

3. In Barred Plymouth Rock fowls the number of visible oöcytes increases with advancing age of the bird, within the age limits of six months and thirty-seven months. The probable explanation of this phenomenon is given.

4. By removing a portion of the ovary and causing it to regenerate, the total number of oöcytes developing to visible size in the lifetime of the bird is caused to increase from 33 to 68 per cent over the number which develop in the normal, unoperated bird. This increase is shown to be statistically significant.

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Resumen por el autor, W. W. Swingle.

La relación de la parte intermedia de la hipófisis con los cambios de la pigmentación en la larva de los anuros.

El autor ha llevado á cabo la transplatación homoplástica y heteroplástica de la parte intermedia de la hipófisis de ranas adultas de las especies *Rana catesbiana*, *Rana clamitans* y *Rana pipiens* en renacuajos de la rana toro de diversas edades y tamaños. Los efectos del injerto sobre el crecimiento y metamorfosis de los animales fueron negativos pero los cambios en la pigmentación a raíz de la transplatación del tejido son muy marcados. Al cabo de veinticuatro horas después del injerto de la parte intermedia intraperitonealmente ó en los espacios linfáticos abdominales las larvas aparecen fuertemente pigmentadas, cambiando su color desde un amarillo claro hasta en color casi negro. El cambio de la coloración se debe á una marcada expansión de los melanóforos de la piel, si bien las células pigmentarias situadas más profundamente en el renacuajo también se dilatan.

La pigmentación exagerada dura mientras el tejido pituitario injertado permanece funcionando y no es reabsorbido. A raíz de la reabsorción del injerto los animales vuelven á adquirir la coloración primitiva normal. El medio ambiente no influye en apariencia sobre los cambios de color que siguen al injerto de la parte intermedia; los cambios se deben á los efectos de los hormonas, que actúan directamente sobre los melanóforos ó indirectamente por intermedio del sistema nervioso. La interrelación posible entre la parte intermedia y otros órganos endocrinos en la formación de pigmento es objeto de discusión en este trabajo, así como los experimentos sobre los efectos de la transplatación de la glándula pineal de los quelonios sobre la pigmentación de las larvas de los anuros. Los puntos discutidos en este trabajo van ilustrados mediante dibujos y fotografías.

THE RELATION OF THE PARS INTERMEDIA OF THE HYPOPHYSIS TO PIGMENTATION CHANGES IN ANURAN LARVAE

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FOUR TEXT FIGURES AND TWO PLATES

The experiments recorded here were begun by the writer in April, 1920, at the Biological Laboratory of Princeton University and some of the principal results mentioned in the present paper were obtained at that time. The experiments were discontinued during the summer to be resumed in September at this laboratory on a much larger scale than was possible before. The earlier experiments were confined to transplanting the anterior and intermediate lobes of the hypophysis of adult *Rana catesbeiana* into immature larvae of the same species, i.e., the grafts were homologous. The pars nervosa and pars tuberalis of the gland were not taken into consideration at that time.

In the earlier work it was observed that transplantation of the anterior lobe of the anuran hypophysis into year-old larvae averaging 85 mm. total length induced a slight increase in the body growth of the animals and marked acceleration of limb growth. In two individuals the fore legs appeared, and there were other evidences of metamorphic changes within thirty days from the date of engrafting the glandular tissue. The majority of the larvae did not go so far as this before the experiments were abandoned. Transplants of the anterior lobe of pituitary were also made on larvae averaging 50 mm. total length, without limbs. The growth rate increased markedly in these younger animals, and there was also rapid growth and differentiation of the hind limbs though the metamorphic changes were not nearly so marked nor so rapid as following transplantation of the pituitary

into older larvae. These results are interesting when it is recalled that under normal conditions the larvae of the bullfrog require approximately two years to complete metamorphosis. Fully differentiated legs do not appear usually until the larvae are almost full-grown in regard to size and over a year old. Hence, when hind limbs are induced to grow to a length of 14 mm. in immature tadpoles of 50 mm. total length by transplantation of the anterior lobe of the hypophysis, and such growth occurs within forty days, it is readily seen that the changes induced are indeed marked. The effects upon metamorphosis following transplantation of the thyroid of adult frogs are very much more rapid, and more marked in every feature of the reaction than those following transplantation of the anterior lobe of the pituitary. Grafts of the anterior lobe "activate" the physiologically inert gland of immature larvae.

These experiments on the transplantation of the anterior lobe of the hypophysis and the thyroid gland are not discussed in the present communication, and are mentioned here in order to show that my experiments along this line, together with those recorded here, were begun simultaneously with and independently of the closely similar work of Prof. B. M. Allen, of the University of Kansas. Professor Allen has recently published a brief statement of his results in *Science* (vol. 52, no. 1342, 1920). Our results are in essential agreement, differing merely in such minor matters as technique, frog species used, and whether homo- or heteroplastic transplants were utilized.

MATERIAL AND METHODS

Allen used normal, pituitariless and thyroidless tadpoles of *Rana pipiens* and made use of homoplastic grafts only; my own material consisted of the neotenuous larvae of the bullfrog with glandular material from several frog species; i.e., the grafts were both homo- and heteroplastic in nature.

The tadpoles used, in the majority of the later experiments, were caught in the vicinity of New Haven and all came from the same pool. The average size was 55 mm., from snout to tip of the tail; most of the animals had minute hind-limb buds. Dur-

ing the course of the experiments, Elodea and other aquatic plants were used as the source of food supply. Before operating, the animals were anaesthetized in a weak solution of chloretone, and after all movement had ceased were transferred to wet towel paper, ventral side up. A small transverse slit through the body wall and peritoneum was made with dissecting scissors, just lateral to the median line above the mesonephros. The glandular tissue to be transplanted was then shoved through the opening with a blunt needle back toward the median line of the body. In many cases the tissue was transplanted in the abdominal lymph sacs. Either method is satisfactory and equally simple. The mortality resulting from such operative procedure is practically negligible. It is a rather striking fact that an operation as severe as an incision into the body cavity should be followed by such rapid recovery and so few fatalities. There is, however, practically no loss of blood, and the wound is very soon closed by a lymph clot that serves to hold the transplanted tissue in place and prevents other foreign substances from entering the body cavity. Recovery from the effects of the chloretone is rapid, and apparently no part of the procedure causes any harmful results. Normal animals used as check controls were treated in a similar way with engrafted brain and muscle tissue instead of glandular substance.

The glandular material was taken from adult frogs of three species, *Rana catesbeiana*, *Rana pipiens*, and *Rana clamitans*, several young specimens of the latter species were also employed.

The hypophysis of an adult frog is readily dissected out by first severing the head from the body just behind the medulla, and then with a pair of sharp-pointed dissecting scissors clipping away the floor of the brain case and exposing the gland. I have found it expedient to cut out the hypophysis and tuber cinereum as a unit in my later experiments, on account of the intimate relation between the pars tuberalis of the hypophysis and this portion of the brain floor.

It is well known to all recent investigators of the pituitary that the gland consists of four parts or lobes, as they are sometimes called, each portion distinct from the others both ontogenetically and histologically. The epithelial part of the pituitary

or that portion which takes origin from the wedge-shaped invagination of cells from the inner layer of oral ectoderm in the early embryo, differentiates during the course of development into three parts: the anterior lobe, the pars intermedia, and the pars tuberalis, first recognized by Tilney ('13) as a distinct portion of the gland. The remaining lobe of the gland, the pars nervosa, is derived from the tip of the infundibulum. It is not within the scope of this paper to discuss at length the anatomical relations of these parts of the hypophysis in adult Anura, especially in view of the recent excellent paper by Atwell ('19). In figure 1 is shown a ventral view of the hypophysis of the bullfrog illustrating the positions of the various lobes.

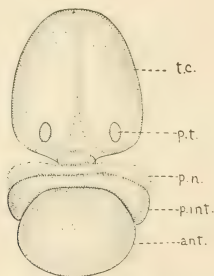


Fig. 1 Ventral view of the hypophysis of an adult *Rana catesbeiana*. *t.c.*, tuber cinereum; *p.t.*, pars tuberalis; *p.n.*, pars nervosa; *p.int.*, pars intermedia; *ant.*, anterior lobe.

The pars tuberalis is seen to consist of two plaques which underlie the lateral thickened portions of the tuber cinereum (infundibulum of many writers, though it is doubtful if the lateral thickened portions of this structure can properly be regarded as such). It is readily seen from the diagram that this portion of the hypophysis is entirely separate from the remainder of the gland.

The anterior lobe is the most conspicuous of the remaining parts of the gland, and paradoxically, instead of being the most anterior as its name implies, it is the most posterior part of the hypophysis in the frog. Its outline is oval, and it is a delicate pink in color, due to the presence of numerous blood vessels.

The pars intermedia is a transverse band of tissue with bulging lateral terminations, closely applied to the anterior lobe. Posteriorly it conforms so closely to the shape of the pars nervosa that it is difficult to make a clean-cut dissection of the two parts under the microscope. The texture and color of the tissue of the two lobes differ to such an extent, however, that it is easy to distinguish them.

OBSERVATIONS

Forty-seven larvae, light greenish-yellow in color, were engrafted intraperitoneally with the pars intermedia of the hypophysis of the three species of *Rana* mentioned. Sixteen hours after the operation, the experimented animals were distinctly darker than the controls, and at the end of forty-eight hours they were nearly black. The controls, engrafted with brain and muscle tissue, retained the normal light-greenish yellow color (fig. 2). The change in color following the operation is very striking, indeed, on account of the color contrast between control and hypophysis-grafted tadpoles, and also because it is so uniform. Any possibility of the environment's playing an active rôle in the reaction was ruled out by the controls, although it is quite true that chloretone tends to cause slight expansion of the melanophores. It was observed that after anaesthetizing the control tadpoles in chloretone the animals turned somewhat darker ten to twenty minutes afterward; this condition lasted but a short time, after which the larvae resumed their normal coloration. The reaction was not at all comparable to the striking effects following transplantation of the pars intermedia (fig. 2).

The darkly pigmented condition persists in the engrafted animals for varying periods, but in none did it last longer than forty days. It has been my experience that the larvae engrafted with the glandular tissue from adult frogs of different species, i.e., heteroplastically grafted, are the first to return to normal coloration. Animals homoplastically grafted tend to retain the dark color for longer periods, though this is certainly not an invariable rule. In the case of heteroplastic transplants, twenty-six days was the maximum and ten days the usual period for the retention of the

dark color. After this there is a gradual (but in some instances a very abrupt) change to the normal greenish-yellow coloration. The average time required for animals homoplastically grafted to regain their usual color is twenty-six days from the date of the operation. The change from almost black to yellow is usually a gradual process, parts of the tadpole body turning yellow, other portions retaining the dark color, thus giving the animal a sort

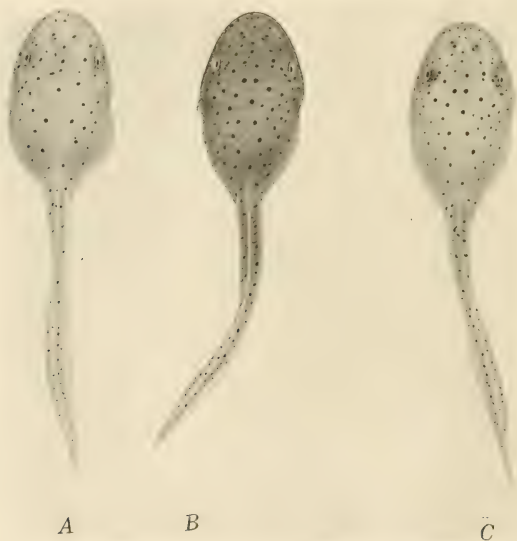


Fig. 2 A, normal control; B, engrafted larvae; C, engrafted animal following resorption of the graft.

of mottled effect. Three cases were observed of larvæ resuming the yellow color overnight from a deeply pigmented condition. In figure 2 the same tadpole is shown darkly pigmented in B, and in C, twelve hours later, it has changed from dark to yellow.

Histological examination of the grafted tissue of such individuals reveals clearly the reason for the resumption of normal coloration in these tadpoles. The graft has, for the most part,

been resorbed; that is, the glandular tissue has disappeared, and nothing remains of the transplant but a mass of connective tissue and blood vessels. On the other hand, in the black individuals the engrafted tissue always shows the presence of secreting glandular cells. The transplanted tissue of the hypophysis in both hetero- and homoplastic grafts evidently is largely resorbed in bullfrog larvae of 55 mm. total length about twenty-six days or less from the date of transplantation. The transplanted tissue is generally found attached to the peritoneum of the body wall and is much more satisfactorily handled for microscopic examination when left attached to a section of the somatopleure than when separated from it. Otherwise, the minute size of the graft renders handling difficult. The implanted *pars intermedia* very soon becomes invaded with blood vessels from the peritoneum. Allen ('20) failed to state whether or not any of his animals resumed normal coloration following transplantation of the *pars intermedia*, nor was any histological examination made to ascertain the fate of the graft. A more detailed report by Professor Allen upon this point is awaited with interest.

Several tadpoles, previously darkly pigmented by reason of the engrafted tissue of *pars intermedia*, but which subsequently resumed the yellow color owing to resorption of the transplant, were reengrafted. The results were always the same, the animals turned very dark for several weeks, then returned gradually to normal coloration. Aside from the more rapid resorption of the grafted tissue of other species of frogs, it apparently made no difference in my experiments, so far as the results were concerned, whether the transplant was homo- or heteroplastic in nature. This is more or less to be expected in grafts involving endocrine glands, owing to the presence of the physiologically active hormone in the glandular substance transplanted. Even though the graft be resorbed, a sufficient amount of the active hormone is generally obtained to bring about a response on the part of the organism.¹ In a recent series of experiments with

¹The pigmentary changes are more marked when the *pars intermedia* and *pars nervosa* are transplanted together, than when the intermediate lobe is grafted alone. The inclusion of the posterior lobe causes a remarkable shrinkage of the larvae due to the diuretic effect upon the mesonephros and consequent loss of turgor by rapid elimination of water.

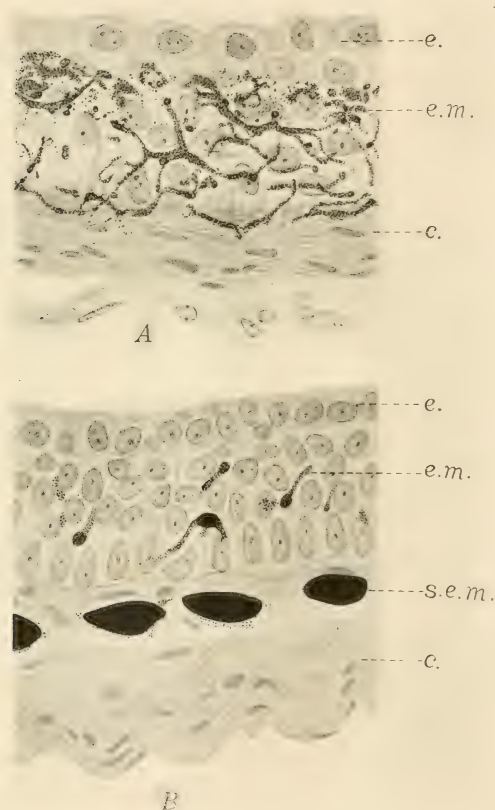


Fig. 3 A, section through skin of engrafted animal. *e*, epidermis; *em*, epidermal melanophores; *c*, stratum spongiosum of corium, containing greatly expanded subepidermal melanophores. B, section through skin of light-colored control. *e.m.*, epidermal melanophores; *s.e.m.*, subepidermal melanophores contracted; *e*, stratum corneum; *c*, corium.

transplanted thyroid glands from animals dead over twelve hours, this point has been well brought out.

A word regarding the various types of pigment cells found in the frog skin may serve in some measure to clarify the discussion to follow. There are several varieties of pigment cells which normally occur in the skin of anurans, and of these there may be distinguished: the melanophores or black pigment cells, leucophores or interference cells, xanthophores, and xantholeucophores or golden pigment cells. Some species of frogs have red pigment.

The melanophores are regarded by the majority of investigators as fixed stellate cells with many irregular branching processes. The pigment is in the form of numerous minute brown or black granules of melanin scattered in the cytoplasm. This type of chromatophore is most abundant on the dorsal surface of the body, and occurs in dense masses in the black spots found scattered over the back and sides of the animal. In sections through the skin (fig. 3 *B*) they are found mostly in the superficial layer of the corium just below the epidermis. A somewhat different type of chromatophore is found in the epidermis; it consists of a cell body with two or more simple processes. Such cells usually lie singly in the epidermis (especially in lightly pigmented individuals), though they may occur in darker animals in sufficient abundance to give the appearance of a reticulum. This type of melanophore is said by Hooker ('14) not to be contractile, though the writer is inclined to question this assertion, especially as regards conditions in *Rana catesbeiana*. On the ventral surface of the tadpole and frog body, these two types of pigment cells are almost absent over considerable areas. The subepidermal melanophores are innervated by motor fibers proceeding along both sympathetic and spinal paths, but, as Laurens ('15) has shown, this type of pigment cell may also respond to direct stimulation without the intermediation of the nervous system. As shown in figure 3 *B*, the subepidermal melanophores present, when contracted, the appearance of small rounded densely staining masses.

According to Smith ('20), the xantholeucophores of the anuran larva form two well-defined layers over the dorsal portion of the

body, each layer being composed of independent cells. The superficial layer lies beneath the epidermal basement membrane, and is separated by a considerable interval from the deeper layer situated just above the corial melanophores. These cells bear guanine and xanthine, and when expanded, give the silvery and iridescent color effects so marked in hypophysectomized tadpoles.

The dark engrafted individuals, when examined under a binocular microscope, showed great expansion of the epidermal and

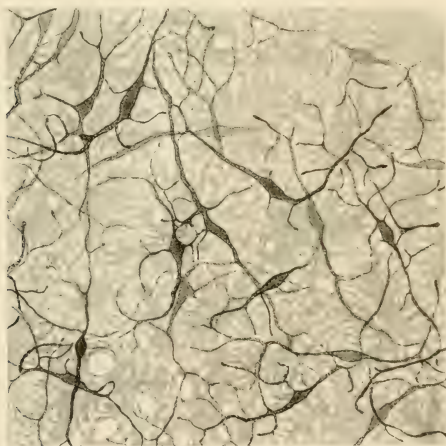


Fig. 4 Surface view of whole mount of skin from engrafted larvae. The two layers of melanophores epidermal and corial are greatly expanded.

subepidermal melanophores of the skin as compared with the normal controls. The degree of expansion of the superficial melanophores of the experimented larvae is striking, the much branched and irregular network of cellular processes apparently forming a dense reticulum. The expansion of the subepidermal pigment cells is so great that an opaque sheet of melanin is found. In the light-colored controls, the melanophores are contracted into dark ovoid masses, and there is no network or reticulum of epidermal melanophores. It is possible, however, to find in any

large culture of bullfrog larvae, where there is unequal distribution of light and shadow, certain individuals with more or less expanded melanophores of both epidermal and subepidermal type. When these dark tadpoles are examined, it is found that the pigment reticulum, though present, is not so dense as in the engrafted animals. In such larvae the dark pigmentation is temporary, and the expansion of the melanophores fluctuates with changes in the environment. Such is not the case with engrafted larvae, for when they turn dark, the expansion of the pigment cells is permanent under all conditions of the environment, so long as the glandular tissue remains intact and supplies the necessary stimulus to the melanophores (fig. 4).

Not only are the subepidermal melanophores expanded to their maximum extent in engrafted animals, but this expanded condition of the pigment cells apparently extends throughout the body (fig. 10). Examination of the deep-lying melanophores, such as are found in large numbers on the lungs, liver, peritoneum, pericardium, and brain membranes, showed that the effect of the engrafted *pars intermedia* is not confined solely to the superficial pigment cells of the epidermis and corium. Pigmentary conditions in the deep-lying organs are not so striking and clear-cut as in the skin, because of the fact that in normal larvae the melanophores of these structures are usually in a more or less expanded condition. If, however, the lungs of normal and experimented tadpoles are dissected out and used as a basis for comparison, it is readily seen that the degree of expansion of the numerous melanophores on these organs of the engrafted animals is greater than that of the normal controls.

These observations are directly in line with those of Allen ('17) on hypophysectomized tadpoles. He found that the animals deprived of the epithelial portion of the hypophysis show a contracted condition of the melanophores throughout the body. That is, when the *pars intermedia* is extirpated, the pigment cells all over the body contract and remain in this condition permanently. Smith, however, finds in his careful study of albinism in tadpoles deprived of the buccal portion of the hypophysis that in young animals the subepidermal melanophores are contracted

as Allen stated, but in older animals the corial sheet of pigment cells appears much as in normal larvae and persistent contraction of the melanophores does not occur. The silvery appearance of the pituitariless animals is due primarily to reduction in number and melanin content of the epidermal melanophores and the persistent expansion of the layers of xantholeucophores.

The contraction and expansion of the melanophores throughout the body following hypophysectomy in the first instance and transplantation of the gland in the second, is to be expected. It would be odd, indeed, if only the superficial melanophores were affected by the hormone, or activating substance of the pars intermedia.

Fixed and stained preparations of the skin and other tissues were employed as a means of checking conditions observed in the living tadpole. Whole mounts and sections of the skin were both used, the skin being taken from the dorsal surface of the body. Because pigmentary conditions are more readily followed in the skin than in other tissues, the discussion is chiefly confined to changes occurring in the epidermal and subepidermal melanophores.

It was observed in sections through the skin and in whole mounts of skin from the experimental animals that the subepidermal pigment cells were greatly expanded, their processes extending like branches of a tree through the stratum spongiosum of the corium. Expansion is so great that the processes of the different cells interlace to form a very dense pigment sheet. In figure 3*A* is shown a section through the skin illustrating this point. The much-branched melanophores are shown cut in various planes by the microtome knife. Figure 3*B* shows the condition presented by the light-colored control larvae. Here the subepidermal melanophores are shown as much contracted masses lying in the corium. Transverse sections are not so good for illustrating expansion and contraction of the melanophores as are surface views of the integument in which the entire cell appears instead of portions of it. Figure 6 shows a photomicrograph of the melanophores of an engrafted animal from a surface view of the skin, or rather a section cut obliquely in such a manner that practically a surface view is obtained, and in figure 5 is shown a

similar view of a normal (light-colored) animal. The difference in the degree of expansion of the pigment cells is obvious. Figure 10 shows the expansion of the deep-lying melanophores in the meninges of the brain in an engrafted specimen.

As previously mentioned, Hooker ('14) states that the epidermal melanophores are non-contractile, and although this may be true of the form with which he worked, it is apparently not the case with young *Rana catesbeiana* larvae. In skin preparations of this species, both in whole mounts and in sections, it is evident that in light-yellow individuals the expanded epidermal type of melanophore is very scarce and difficult to find. This is exceptionally well shown in whole mounts of the skin. The subepidermal cells are found to be contracted and present the appearance of black balls, with little indication of cellular structure. On the other hand, whole mounts of the skin of darkly colored engrafted tadpoles show two distinct layers of chromatophores, a superficial and a deep, lying at different levels. The upper layer or epidermal melanophores form a definite network, with the cell processes of neighboring cells in contact, thus giving the appearance of a reticulum (fig. 6, also fig. 4). The deeper layer of pigment cells (subepidermal) are greatly expanded and present the familiar 'mossy' appearance characteristic of this phase of the cell. It seems to the writer that, unless the epidermal melanophores possess the power of contractility, it is otherwise difficult to explain why light-colored animal show so few of these cells, while in engrafted individuals they are not only very numerous, but their processes give the appearance of an anastomosing network. In sections through the skin, similar conditions are seen; the epidermal melanophores are numerous, with long cell processes in deeply pigmented engrafted larvae, but very sparsely scattered in the epidermis of the light-colored controls. Judging from conditions in my material, it seems that these pigment cells are more or less contractile, though evidently not to the extent that the larger, subepidermal melanophores are. They apparently increase in number, or at any rate become visible with elongate processes in engrafted animals, thus playing an active part in the color change following transplantation of the *pars intermedia*.

Laurens, working on *Amblystoma* larvae, states that the epidermal melanophores of this form expand and contract, and hence appear to play an active rôle in pigmentation changes. The recent work of Smith likewise indicates that this type of pigment cell is contractile.

In this connection it is interesting to note that following hypophysectomy the resulting albino larvae appear to lose their epidermal melanophores to a large extent, and according to Atwell ('19) the few that remain contain only a small proportion of the normal number of pigment granules found in similar cells of the control larvae. Allen ('17) thinks that following removal of the pituitary, the epidermal pigment cells migrate to deeper positions in the animal, a conclusion with which Smith disagrees. However this may be, transplantation of the *pars intermedia* causes the appearance of large numbers of this type of melanophore in the skin of the bullfrog tadpole, though there can be little doubt that the dark color of the engrafted animals is due chiefly to the subepidermal melanophores. It is highly improbable, as Smith has shown, that the corial chromatophores play an important rôle in the picture of albinism resulting from hypophysectomy, no matter whether they are contracted or expanded, because they are completely masked by the broad and persistent expansion of the layers of xantholeucophores above them.

Transplantation of the *pars intermedia* brings about contraction of the xantholeucophores, thus enhancing the 'darkening' of the larvae. In the present discussion we are not concerned with this type of pigment cell.

DISCUSSION

The rôle of the hypophysis in producing pigmentary changes in anuran larvae has received considerable attention since the publication of the work of Smith ('16) and Allen ('17) on hypophysectomy in the frog embryo. Both of these investigators, working independently, succeeded in ablating the epithelial anlagen of the hypophysis at the time of its first appearance as a wedge-shaped invagination of cells from the oral ectoderm. Within a few days following the operation the tadpoles lose their

normal dark coloration and turn silvery white, or, according to Smith, they become albinos. The pigmentary change is characteristic and striking.

Smith and Allen hold somewhat different views regarding the nature of the change in pigmentation following hypophysectomy. Smith considers the silvery appearance of the larvae as being due primarily to a reduction in number and melanin content of the epidermal melanophores, and to persistent expansion of the xantholeucophores. The expansion of the latter gives the tadpole its silvery appearance. He states that the corial melanophores are equally expanded in the albinos and the control animals.

Allen, on the other hand, believes that the albino effect is due to the migration of the epidermal pigment cells to deeper positions in the body and, moreover, that all the melanophores throughout the body are contracted following ablation of the epithelial portion of the pituitary.

Neither of these investigators had any good evidence as to just what portion of the hypophysis is responsible for the change in color following extirpation, for in their experiments three parts of the hypophysis were removed, anterior lobe proper, pars intermedia, and the pars tuberalis. It remained for Atwell ('19) to show that it is the lack of the pars intermedia that is responsible for the albino appearance of the hypophysectomized larvae. He extirpated the hypophysis of the frog embryo and, following the 'silvering' of the tadpoles, placed them in dilute extract of pars intermedia of beef pituitary. The animals soon underwent a striking color change from silvery to dark, in the latter condition closely resembling normal tadpoles. When returned to fresh water the darkened larvae regained the silvery appearance.

Atwell interpreted his results as indicating that the 'silvering' of the hypophysectomized larvae was due to a prolonged and sustained contraction of the pigment-bearing cells, owing to loss of the pars intermedia. Smith, in his recent monograph, puts a different interpretation on Atwell's work. He confirms Atwell's observations that the corial melanophores expand in solutions of pars intermedia, but he noted further that not only do the larvae become darker, but they almost completely lose their metallic

iridescent appearance so characteristic of the picture of albinism. That is to say, the xantholeucophores are contracted and the silvery appearance is lost, the animal appearing dark like normal tadpoles. Hence, the picture of albinism is not so much due to contraction of the corial melanophores as to expansion of the overlying layers of xantholeucophores. This interpretation of Smith's has recently been controverted by Atwell.

In a recent communication ('20) Allen has reported a very brief summary of his results with transplanting the pars intermedia of the adult frog. He says: "Normal tadpoles into which the intermediate lobe is engrafted become much more darkly colored than the controls, while those which have been made to turn white as a result of removal of the anlage of the hypophysis exclusive of the posterior lobe are made to change back from white to black when the intermediate lobe is engrafted into them."

In view of these experiments and those recorded here, it can be regarded as a well-established fact that in anuran larvae the pars intermedia secretes a substance which exerts a powerful effect upon the melanophores. The absence of the hormone results in permanent contraction of many of these cells, and expansion of the xantholeucophores, its presence in large quantities such as follows transplantation of the glandular tissue, causes maximum expansion of the epidermal and corial pigment cells and contraction of the bearers of guanine and xanthine.

It has long been known that many conditions affect the melanophores of the frog skin; a list of such agencies includes temperature, electric currents, variations in the intensity of light, and many chemical substances aside from hormones. The effect of such stimuli, however, is temporary and lasts only so long as the melanophores are subjected to the influence of the agent. On the other hand, in the case of the hormone of the pars intermedia of the hypophysis, we are dealing with an internal regulating agent of high potency, the effects of which are continuous, and not to any extent influenced by changes in the environment. For instance, engrafted larvae which have turned dark do not respond by color change, to light stimulation, and neither do albino larvae deprived of the pars intermedia. Smith has shown that the cor-

ial melanophores of such larvae expand under the stimulus of light, but the xantholeucophores are not affected, consequently the 'pituitariless' animal remains silvery.

Many of the agents mentioned exert their influence upon the melanophores through the intermediation of the nervous system. In the transplantation experiments recorded here it is probable that the hormone acts directly upon the melanophores themselves by way of the blood stream, and not through the intermediation of the nervous system, though the latter possibility is not ruled out. However, if, as has been clearly shown by Laurens ('15), other agencies are capable of acting directly upon the melanophores, it is very likely that the hormone of the pars intermedia does likewise.

It is interesting to note that the secretion of another endocrine gland, arising as an outgrowth from the dorsal surface of the diencephalon, has a marked effect upon the melanophores of anuran larvae. McCord and F. P. Allen ('17) have clearly demonstrated that the pineal gland of mammalia, when administered in various ways to anuran larvae, brings about contraction of the subepidermal melanophores, thus causing the tadpoles to assume a condition simulating the silvery appearance characteristic of hypophysectomized animals. The effects of this gland are temporary and soon disappear.

Recently the writer transplanted the pineal gland of reptiles (*Chelonia*) into darkly pigmented larvae of *Rana clamitans*, and obtained rather interesting results. Within thirty to forty minutes following implantation of the gland into the body cavity, the larvae turned distinctly lighter in color, i. e., from a dark brown to a light greenish-yellow. The light color persisted for several hours, when the animals gradually resumed normal coloration. Mammalian pineal-gland substance (desiccated) in form of pills was injected into the body cavity with similar results. These experiments confirm the previous work of McCord and Allen (F. P.) upon feeding mammalian pineal substance to tadpoles, though the color change following implantation or injection of the gland in my larvae was not nearly so striking as that described by these investigators when the tissue is fed.

It has been demonstrated by several investigators that ablation of the pineal apparatus of amphibian embryos apparently has little effect upon the growth and development of the pigmentary system. It should be noted, however, that this gland when extirpated in very young larvæ has the power to regenerate, hence it is possible that it may play a more important rôle in inducing pigment changes than we think. It is certain that the pineal gland exerts no such marked and continuous power over the melanophore system as does the pars intermedia.

It is interesting to note that the removal of the pars intermedia causes persistent expansion of the xantholeucophores, and that implantation brings about their contraction along with persistent expansion of the melanophores. Here we are dealing with a differential effect upon the pigmentary system of a single gland, maximum expansion of the melanophores when an oversecretion of pars intermedia is obtained, as, for instance, by transplantation experiments, maximum expansion of the xantholeucophores when the pars intermedia is lacking. Taking these facts into consideration, it is possible that instead of dealing with differential effects of a single gland upon the tadpole's pigmentary apparatus, we may be observing the effects of interaction between two internal secretory mechanisms. It seems rather improbable that the mere absence of the pars intermedia is capable of bringing about maximum and continuous expansion of the xantholeucophores, such as occurs in 'pituitariless' frog larvæ. It is improbable that the 'absence' of anything could act as a stimulus of the strength required to produce this effect. Is it not possible that the ablation of the pars intermedia has released some other active agent within the body from an inhibitory influence exerted by this portion of the hypophysis? This is only a suggestion, and should be regarded as such.

In this connection it is of paramount importance to know which condition of the chromatophore, the expanded or contracted, is to be regarded as the active or passive phase. If, as assumed by some (Spaeth, '16, and Laurens, '15), the expanded condition of the pigment cell is the passive phase then the persistent expansion of the xantholeucophores following hypophysectomy in the

embryo frog is accounted for, but not the diminution in number and melanin content of the epidermal melanophores. On the other hand, if, as stated by many workers on pigment cells, the expanded condition is the active phase, then we are faced by a difficult problem when considering the albino, for unless the alternative proposition, that some other internal secretory mechanism is at work along with the pars intermedia, is adopted, we must assume that an 'absence' can bring about very positive differential results within a single system.

In conclusion, the writer takes this opportunity to express his obligation to Miss Julia E. Lovett, of the Osborn Zoological Laboratory, for the excellent drawings.

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PLATE 1

EXPLANATION OF FIGURES

Microphotographs

- 5 Contracted condition of subepidermal melanophores of normal larvae. Surface view from whole mount of skin. $\times 700$.
- 6 Expanded melanophores giving the appearance of a reticulum. Surface view from section cut on bias. $\times 700$.

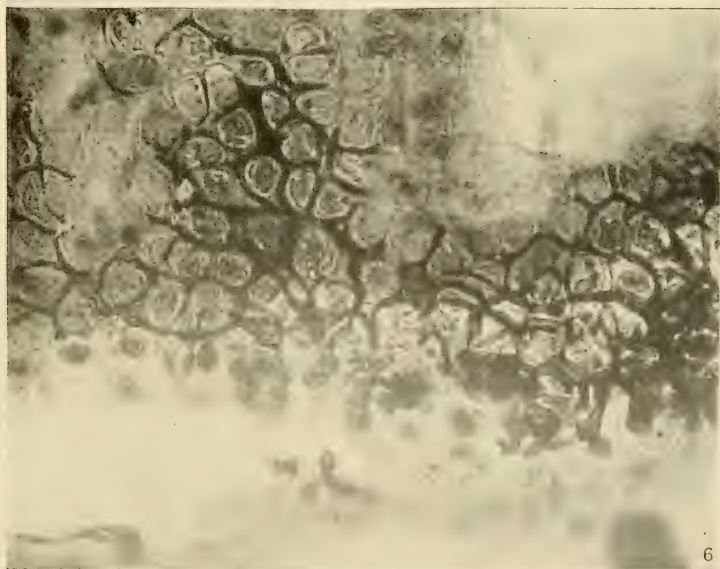
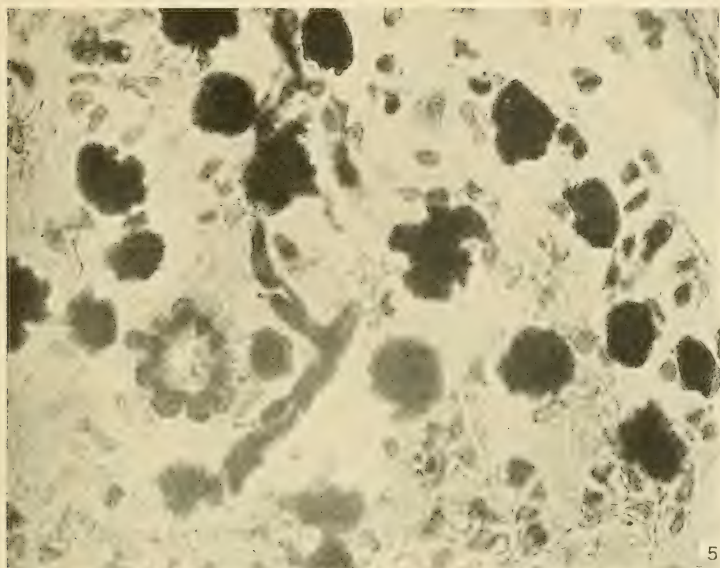
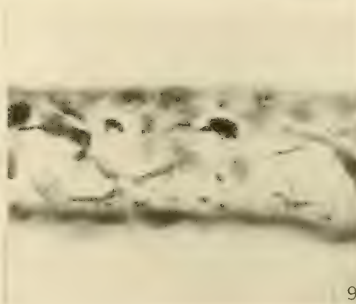
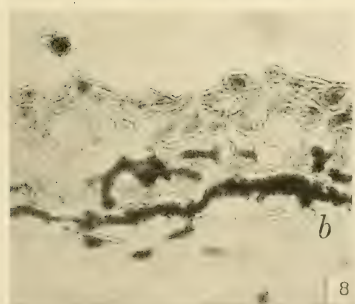
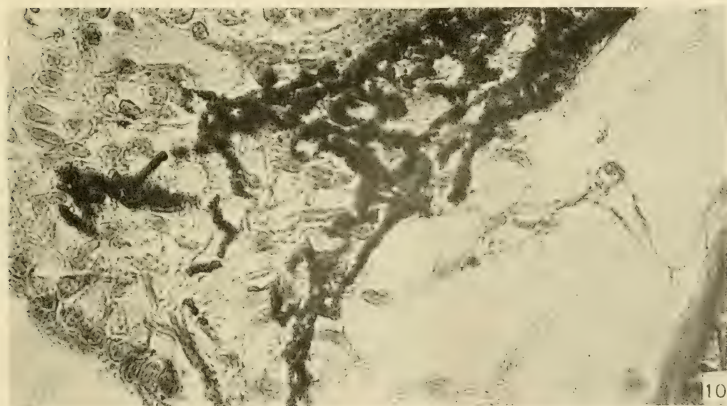
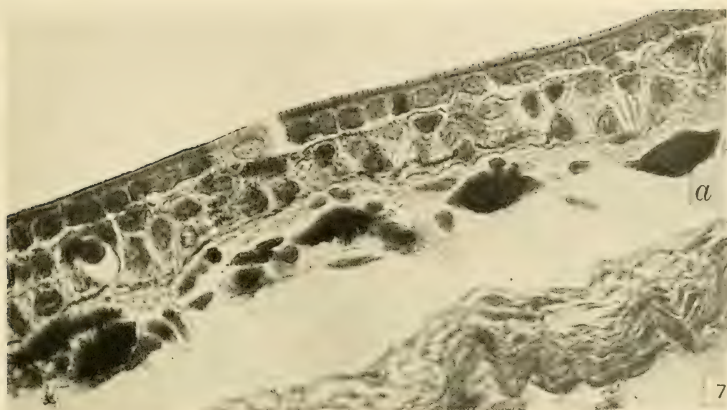


PLATE 2

EXPLANATION OF FIGURES

- 7 Section through the skin of control, showing contracted melanophores at *A*. $\times 700$.
- 8 Section through skin of engrafted individual. Expanded epidermal melanophore above the subepidermal pigment cells at *B*. $\times 700$.
- 9 Expanded melanophores in skin of engrafted animal. $\times 700$.
- 10 Expanded melanophores in meninges of brain of engrafted animal.



Resumen por el autor, Joseph Hall Bodine.

Algunos factores que influyen sobre el contenido de catalasa en los organismos.

Los resultados expuestos en el presente trabajo demuestran que el contenido de catalasa en los animales (saltamontes, gusano de luz, escarabajo de la patata (*Leptinotarsa*)) disminuye á medida que la edad aumenta. La cantidad de anhídrido carbónico producido y el contenido de catalasa parecen variar en la misma dirección, pero en los animales hibernantes (saltamontes) no existe dicha relación. El contenido de catalasa disminuye durante la inanición. El autor hace notar que es necesario poseer más datos cuantitativos para demostrar la existencia de una relación definida entre el contenido de catalasa y la cantidad de anhídrido carbónico producido por los organismos.

Translation by José F. Nonidez
Cornell Medical College, New York

SOME FACTORS INFLUENCING THE CATALASE CONTENT OF ORGANISMS

JOSEPH HALL BODINE

Zoological Laboratory, University of Pennsylvania

THREE FIGURES

In recent years attention has been directed to the presence of the enzyme catalase in organisms and various interpretations of its functions advanced. Most striking are the properties attributed to it by Burge and Burge.¹ These authors assume that the presence of catalase determines the rate of oxidations occurring at different stages in the life-cycle of an organism, e.g., "*the increase in the respiratory metabolism or oxidation in youth and decrease in old age are attributed to the increase in catalase in the young and its decrease in the aged.*"

Various experiments have been carried out by the author on the catalase content and the rates of carbon-dioxide output of grasshoppers and other insects to see in how far such a theory applies in the case of these organisms. The results herein presented show, 1) the catalase content of animals of different ages; 2) the relation between the rates of carbon-dioxide output and catalase content; 3) the effect of hibernation on catalase content, and, 4) the effect of starvation on catalase content. These will be discussed in the order given.

METHODS

Catalase determinations were made by the method essentially similar to that described by Burge.² Diluted commercial hydrogen peroxide was used—the same sample for experimental and normal animals in each case. Readings were made at five- and ten-minute intervals, and the rates of oxygen per gram per minute calculated from the averages thus obtained. Carbon-

dioxide determinations were made by the barium-hydrate-titration method of Lund.³

The grasshoppers used (*Chortophaga viridifasciata*) were caught in the vicinity of Philadelphia and kept under the usual laboratory conditions and fed grass. The other organisms used fireflies and potato-beetles—were brought into the laboratory and determinations made at once.

Temperature and other laboratory conditions were kept as nearly constant as possible throughout any series of observations.

CATALASE CONTENT OF ANIMALS OF DIFFERENT AGES

It has been found by the author⁴ that for grasshoppers an increase in body weight accompanies increasing age up to the last instar, hence, heaviest animals are the oldest. Figures 2 and 3 show graphically the relative amounts of catalase contained in grasshoppers of different body weights and ages. Youngest animals tend to have the highest catalase content with a gradual decrease in amount to a minimum for the oldest individuals. Figure 1 shows a similar condition in fireflies taken at various intervals during the season. Experiments carried out on potato-beetle larvae and adults gave similar results, thus agreeing with those reported for these forms by Burge and Burge.¹

From the results of these experiments one can reasonably conclude that the amount of catalase an animal contains decreases progressively during its life-cycle up to a minimum in old age.

RELATION BETWEEN RATES OF CO₂ OUTPUT AND CATALASE CONTENT

Evidence has been presented by the author⁴ which shows that the rates of CO₂ output for grasshoppers are highest for younger and lowest for older individuals. It has been pointed out in the preceding paragraph that the catalase content of these animals also apparently varies in the same direction.

Figure 1 shows that the rate of CO₂ output and catalase content for fireflies seems to bear a similar relation.

From these results, however, it does not necessarily follow that a parallelism between CO_2 output and catalase content exists, since by varying the rates of CO_2 output by external factors, such as temperature, no appreciable changes in the catalase content are brought about. A possible objection, however, to such an experimental procedure can easily be eliminated by taking advantage of the natural 'hibernating' habit of this grasshopper, which lives normally as a nymph throughout the

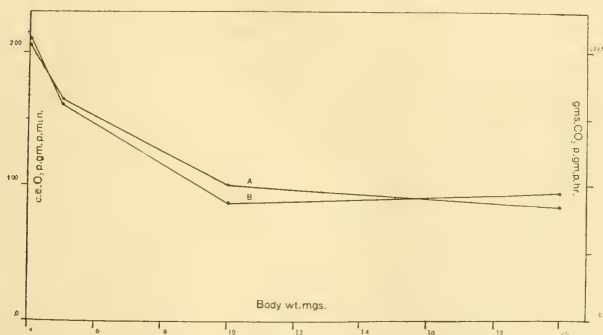


Fig. 1 Curves show the rates of CO_2 output per gram body weight per hour and also the cc. of O_2 evolved per gram body weight per minute for fireflies. A, catalase; B, CO_2 . Abscissas represent body weights in milligrams. Ordinates at the right, grams CO_2 per gram body weight per hour. Ordinates at the left, the number of cubic centimeters of oxygen obtained per gram body weight per minute. Based on some fifty separate determinations. For further explanation see text.

winter. During 'hibernation' the animal becomes extremely sluggish, and metabolic activity must also take place at a greatly reduced rate. Determinations of CO_2 output at room temperature ($20^\circ\text{C}.$) show that the rate of output for hibernating forms (0.0005 to 0.0008 gram CO_2 per gram per hour) is decidedly lower than that for individuals of the same body weights which are not hibernating (0.0018 to 0.0025 gram CO_2 per gram per hour).

If the theory is correct, that catalase content and CO_2 output are definitely related, these hibernating individuals ought to show greatly reduced amounts of catalase. Figure 2, however, shows that the catalase contents of hibernating and growing individuals of the same body weights are practically identical. Results somewhat similar to the above have been reported by Stehle and McCarty⁵ for cats and rabbits.

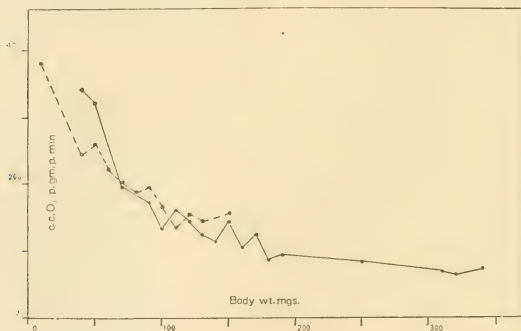


Fig. 2 Curves show the relative amounts of catalase in normal and hibernating grasshoppers of different weights. Solid line, normal animals. Broken line, hibernating animals. Abseissas represent body weights in milligrams. Ordinates represent number of cubic centimeters of oxygen obtained per gram body weight per minute. Based on 315 separate determinations.

EFFECT OF STARVATION ON CATALASE CONTENT

Inasmuch as Burge and Neill⁶ have shown that starvation in higher forms results in a decrease in catalase content, it is of some interest to see in how far such results can be produced in insects.

Groups of grasshoppers were starved for varying periods of time up to 144 hours and catalase determinations made at twenty-four-hour intervals. Determinations on normal animals (those fed) and experimental ones were carried out at the same time, using the same sample of peroxide.

Figure 3 shows graphically the results of such experiments. In general it is found that catalase content falls during successive periods of starvation. Such a result is quite in agreement with that of the above-mentioned authors.

It has also been pointed out⁴ that during starvation in grasshoppers the rate of CO_2 output decreases during successive periods of starvation. Here again as in the preceding paragraphs



Fig. 3 Curves and points show the relative amounts of catalase in normal grasshoppers and in those starved for varying periods of time. Abscissas represent body weights in milligrams. Ordinates represent number of cubic centimeters of oxygen obtained per gram body weight per minute. Based on some 150 separate determinations. For further explanation see text.

one cannot necessarily conclude that this apparent parallelism between the CO_2 output and catalase content shows the two to be definitely related. Much further quantitative data seem to be necessary before such a theory can be definitely established.

SUMMARY

1. Catalase content of insects (grasshopper, firefly, potato-beetle) decreases with increasing age and body weight.

2. The rate of CO_2 output and catalase content in normal animals seems to vary in the same direction, but in 'hibernating' forms a marked decrease in CO_2 output takes place with no corresponding change in catalase content.

3. Starvation results in decreased amounts of catalase.

4. Further quantitative data regarding catalase content and CO_2 output are required before any definite relationship between the two can be established.

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Resumen por el autor, S. O. Mast.

Reacciones hacia la luz en las larvas de las ascidias *Amaurocium constellatum* and *Amaurocium pellucidum*, con especial mención de la orientación fótica.

Los "renacuajos" de *Amaurocium* se orientan con bastante precisión. Cuando emergen de las colonias son fuertemente foto-positivos, pero permanecen positivos solamente durante unos instantes, pasados los cuales se transforman en fotonegativos. Si se reduce rápidamente la luz, los ejemplares en estado de reposo, tanto los positivos como los negativos, responden haciéndose activos y los ejemplares activos responden cambiando su dirección de locomoción, los positivos girando hacia el abocular y los negativos hacia el lado del ocular. El aumento de la iluminación no produce efecto sobre los ejemplares en estado de reposo, á pesar de la rapidez y extensión, pero los ejemplares activos responden girando hacia el lado abocular. Si se cambia gradualmente la iluminación en cualquier dirección no se obtiene respuesta, á pesar de la extensión del cambio.

Todas las reacciones fóticas se deben probablemente á cambios de la iluminación de las terminaciones nerviosas en la superficie interna de la copa pigmentada del ojo. La orientación es el resultado de una ó más reacciones de choque causadas por la sombra y la iluminación alternativas de las terminaciones del nervio óptico á causa de la rotación sobre el eje longitudinal. En los ejemplares negativos la primera produce el giro de la cola hacia el ocular, mientras que la segunda la hace girar hacia el lado abocular; en los ejemplares positivos sucede precisamente lo contrario. Una de las series de respuestas dirige al organismo lejos de la luz, la otra hacia el manantial luminoso. Después que se han orientado, los renacuajos, la retina queda continuamente iluminada de un modo uniforme, cerando las reacciones producidas por el choque. • Permanecen orientados porque tienden á moverse siguiendo un trayecto recto cuando no son estimulados.

REACTIONS TO LIGHT IN THE LARVAE OF THE ASCIDIANS, AMAROUCIUM CONSTELLATUM AND AMAROUCIUM PELLUCIDUM WITH SPECIAL REF- ERENCE TO PHOTIC ORIENTATION¹

S. O. MAST

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TEN FIGURES

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INTRODUCTION

It is well known that the larvae of many aquatic animals respond to light and it is generally assumed that their response facilitates distribution. There is, however, practically nothing known regarding the precise nature of these responses.

¹ I am indebted to the Marine Biological Laboratory of Woods Hole, Massachusetts, for working facilities and to Dr. Caswell Grave for calling my attention to the photic responses of the ascidian larvae and very generously supplying me with some of his material. Doctor Grave has made a very thorough study of the structure and habits of the larvae of the two species used in this work, and the descriptions in this paper, in so far as they refer to structure and habits, are based very largely upon his work, some of which is published (Grave, '20), some in press, and some in the process of preparation for publication.

In his studies on *Amaroucium* ('20), Grave found that when the larvae leave the colonies they orient and swim fairly directly toward the source of light, being strongly photopositive, that they remain positive for only a few moments and then become negative, after which they remain so until they become attached and begin to metamorphose. He maintains that quiescent individuals respond to sudden reduction in luminous intensity by becoming active and that the eye functions as a light-receptor.

In this paper we shall deal primarily with the nature of the photic responses and the process of orientation in the larvae of *Amaroucium constellatum* and *pellucidum*.

The process of orientation has been fairly thoroughly investigated in various organisms, ranging from relatively simple plant structures to highly complicated animals, such as arthropods and vertebrates. The ascidian larva, however, with but one simple eye, asymmetrically located, constitutes a type in which this process has heretofore received no attention.

The results obtained in the study of the larvae of *Amaroucium* have a direct bearing on the theories of orientation, concerning which there is still much diversity of opinion. This matter will be discussed briefly at the close of this paper and more extensively in another paper dealing with the process of orientation in insects.

MATERIAL AND GENERAL BEHAVIOR

The two species of *Amaroucium* which were studied are sessile colonial ascidians, each colony consisting of numerous zooids. The colonies during the summer months produce numerous embryos which are liberated as free-swimming larvae; these become attached and develop into new colonies. The larvae closely resemble amphibian tadpoles in form and they are usually known as tadpoles. They consist of an ellipsoidal body somewhat flattened and a broad tail about twice as long as the body. The body contains among other structures a static organ and an eye. The eye is located at the base of the tail near the surface of one of the narrower sides of the body (fig. 1). This side or edge of the body will be referred to as the ocular side and the opposite

surface as the abocular side. These two sides continue posteriorly in the two flat surfaces of the tail.

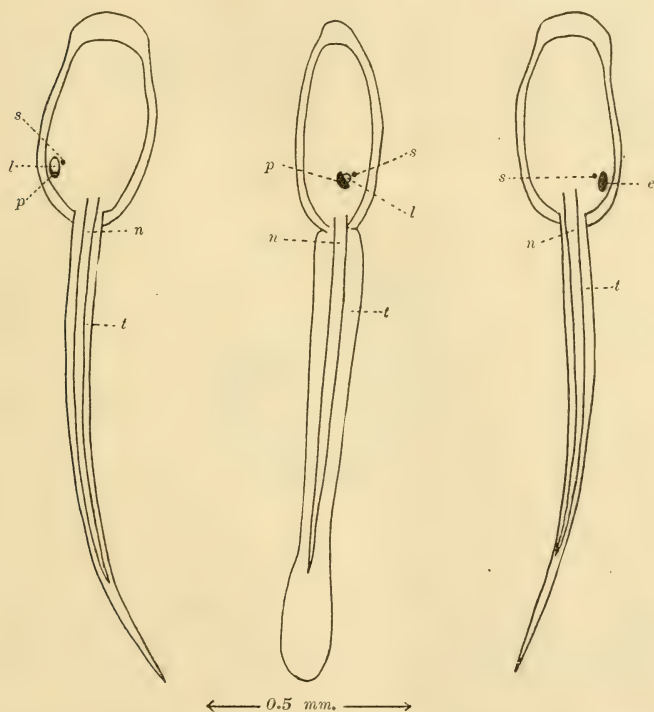


Fig. 1 Camera outlines of tadpole of *Amaroucium pellucidum* as seen from three different surfaces. *t*, tail; *n*, notocord; *e*, eye; *l*, lens; *p*, pigmented eye-cup; *s*, statolith; *mm*, projected scale.

The eye consists of a cup-shaped pigmented structure partially surrounding a hyaline lens structure (fig. 2). The pigmented cup contains a number of pores through which nerve processes pass, ending on the inner surface. The opening of the cup

containing the lens, faces one of the flattened surfaces of the body. When this surface is up the eye is to the left and when it is down it is to the right of the median line (fig. 1).

The tadpoles of *Amaroucium constellatum* and *A. pellucidum* are essentially the same in form and in structure in so far as it concerns behavior. They differ, however, greatly in size, *constellatum* being approximately 2.25 mm. and *pellucidum* 1.5 mm. long.

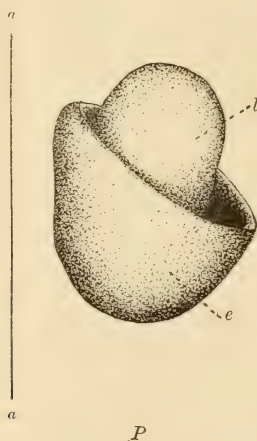


Fig. 2 Eye of tadpole of *Amaroucium constellatum* after a camera outline by Grave from a living specimen. *e*, eye-cup; *l*, lens; *a-a*, longitudinal axis of body; *P*, posterior surface.

Colonies kept in running sea-water in the laboratory produce larvae for a number of days. These are liberated throughout the entire day, but not in abundance except in the morning about sunrise when they can be collected in fairly large numbers at the surface of the water on the side of the jar facing the light. There is thus a certain periodicity in the liberation of the tadpoles. The factors involved in this periodicity have, however, not as yet been definitely ascertained.

During the free-swimming period the tadpoles are first for a short time strongly photopositive and geonegative. Then they become photonegative and somewhat later geopositive and remain so until they become attached, after which they metamorphose and divide vegetatively to form zooids and colonies. Thus each tadpole may develop into a new colony. The free-swimming period is considerably longer in *pellucidum* than in *constellatum*. In the former it varies with different individuals from twenty minutes to twenty-four hours, in the latter from ten to one hundred minutes.

In swimming the tadpoles of *Amaroucium* continuously rotate on the longitudinal axis clockwise as seen from the rear. The forward movement is produced, as in amphibian tadpoles, by lateral strokes of the tail. Rotation is probably produced by a twist in the tail during the lateral strokes, owing to the arrangement of the muscle fibers. The movement of the tail, locomotion and rotation on the longitudinal axis are normally so rapid that details concerning these processes can be ascertained only in specimens in which the activity has been artificially greatly reduced, as, e.g., by reducing the temperature or by confinement to a narrow space. In specimens which swim slowly it can be clearly seen that the tail still vibrates very rapidly, so that there are numerous lateral strokes for every complete rotation on the longitudinal axis. I am not certain, however, that this holds for specimens swimming freely through the water.

In the laboratory, especially in small quantities of water, a few drops, e.g., there are alternate periods of rest and activity. The tadpoles swim rapidly about for a few moments, then come to rest, usually in contact with some object or the surface film, remain a few moments, then without any observable change in the environment they suddenly begin to swim about again, continue for a few seconds, then come to rest, etc. If the light is rapidly decreased, practically all individuals which are at rest become active at once and swim about rapidly; but if it is increased there is no observable response.²

² This was discovered independently by Doctor Grave and the author.

Light of various intensities, including direct sunlight, was repeatedly flashed upon quiet specimens without any indication of a response. An increase in light may, however, under certain conditions act as a stimulus, as we shall show presently.

NATURE OF RESPONSE TO DECREASE IN ILLUMINATION

The nature of the response in the tadpoles of *Amaroucium* to reduction in illumination depends upon whether or not they are at rest when the reduction occurs. If they are at rest they respond by immediately becoming active, as previously stated. If they are not at rest they respond, as will be shown presently, by changing the position of the tail without any apparent change in activity. Thus the response to reduction in illumination or shadows may consist in activation or merely in a change in the position of the tail.

In studying these responses the light was reduced in various ways, e.g., by changing the position of the mirror under the stage of the microscope, by inserting an opaque object between the source of light and the organisms, or by changing the distance between them and the source of light. The last method mentioned was extensively used in the dark-room, where a lamp of any desired power could be moved rapidly toward or from the microscope a distance of over 300 cm. on a track so arranged that the lamp in all positions delivered on the stage of the microscope or binocular a well-defined horizontal beam of light. Thus the illumination could be decreased or increased rapidly or slowly by any desired amount without changing the direction of the rays.

a. Resting specimens

The activation response in resting specimens may consist in rapid locomotion or it may consist in one or more strokes of the tail without locomotion. If there is more than one stroke, the tail usually strikes in one direction a number of times in succession and then in the opposite direction, after which it again strikes in the former direction. These differences in response are, however, differences in degree rather than in kind.

If the response is strong, it results in locomotion; if it is weak, it does not. In older specimens, especially after they are attached and begin to metamorphose, it often consists of but a single stroke of the tail. In younger ones it ordinarily consists of rapid vibration of the tail, which usually results in locomotion.

The rate of locomotion induced by shadows is not appreciably greater than it is normally, and the period of activity under the two conditions is also essentially the same. That is, if the tadpole starts to swim without external stimulation, it continues to swim at approximately the same rate and for approximately the same length of time as it does when it starts in response to a reduction in illumination. Moreover, the periods of activity induced by reduction in illumination are largely if not entirely independent of the illumination following the stimulation. The former conclusions are based upon numerous observations without actual measurements, the latter upon observations with measurements. The results obtained in two series of measurements are presented in table 1. By referring to this table, it will be seen that there is great variation in the length of the periods of activity in different individuals and in the same individual at different times, but that the average period of activity is probably independent of the illumination following the stimulation which induces activity, it being 11.60 hm.³ in the lower and 13.38 hm. in the higher, which is a fairly close agreement, considering the great variation in the periods and the small number of results entering into the compilations.

These facts seem to show conclusively that the reduction in luminous intensity initiates a process which continues without further stimulation, a process of the 'all-or-none' type. This conclusion is further supported by the fact that there is a threshold. No apparent effect is produced by a reduction in light until there has been a given reduction. Then there appears to be a maximum effect regardless of any further change. This is clearly indicated by the results of the following observations:

1. On September 4th a tadpole of *Amaroucium pellucidum* attached under a cover-glass was observed under the microscope

³ hm., abbreviation for hundredth-minute.

in front of a window in the dark-room. This window could be rapidly closed and opened by means of an opaque shutter carried on a horizontal track. The specimen was allowed to come to rest with the window entirely open. By observing the effect of rapidly closing the shutter to various extents, the minimum reduction in illumination necessary to produce a re-

TABLE I

Indicating the time in hundredth-minutes that Amaroucium tadpoles remain active after having been stimulated by sudden reduction in illumination

	REDUCED ILLUMINATION CONTINUED AFTER STIMULATION	REDUCED ILLUMINATION FOLLOWED BY INCREASE IN ILLUMINATION TO NORMAL
Specimen A	38.0	27.5
	35.0	82.2
	20.0	25.5
	17.6	30.8
	19.6	17.2
Specimen B	14.0	6.0
	8.4	8.2
	3.2	3.2
	4.4	2.0
	2.8	12.4
	3.0	3.0
	3.4	5.2
	5.2	5.0
	28.8	2.0
	2.0	2.0
	4.0	1.8
	4.4	4.4
	9.0	3.4
	3.8	5.0
	5.4	20.8
Total average.....	11.60	13.38

sponse was approximately ascertained and the magnitude of the response under minimum stimulation noted. Then, beginning each time with the shutter entirely open, the illumination was variously reduced, but always much beyond the minimum necessary to produce a response. Under each of these reductions the magnitude of the response was ascertained. This could be fairly accurately done by noting the relation between the tip of

the tail, when the lateral stroke of which the response consisted, was at its maximum, and various particles of debris in the preparation. There was no observable difference in the magnitude of the response induced by the reduction of luminous intensity varying from a minimum of approximately 10 per cent to a maximum of practically 100 per cent. Moreover, in numerous observations no difference was discovered in the response produced by a sudden reduction from direct sunlight to very weak illumination and that produced by reduction from weak diffused daylight to the same weak illumination. If there is any difference, it certainly is not proportional to the difference in the reduction in illumination under the two conditions which obtained in the experiment.

In the experimental observations presented above and in numerous other observations it was very evident that it requires to induce an activating response a certain reduction in the amount of light energy received by the organism. The minimum amount necessary was not accurately ascertained, but it was observed that it is under certain conditions relatively very small. For example, in one experiment a tadpole of *Amaroucium constellatum* exposed in a beam of light of approximately 1000 m.c. responded repeatedly to a shadow produced by passing a finger rapidly through the beam. Thus it required, to induce the activating response, a reduction in energy of probably not over 10 m.c. sec. Whether or not the minimum (threshold) amount of reduction in energy is under certain conditions quantitatively constant regardless of luminous intensity in accord with the Bunsen-Roscoe law has not been ascertained. It is, however, clear that there are conditions under which no such quantitative relations hold, for it has been conclusively demonstrated, as the following results show, that the response in question depends upon the time-rate of change in illumination, that no matter how great the reduction in light energy may be the reaction does not follow if the reduction is sufficiently gradual.

2. In attached specimens of both species it was repeatedly observed that there was no response if the lamp on the track in the dark-room was slowly moved away from the organism.

Thus it was found that while rapid movement of a 500 c.p. lamp from 40 cm. to 50 cm. from the organisms produced activating responses, slow movement from 40 cm. to 400 cm. produced no responses. One experiment is particularly interesting in this connection. Under the binocular, twelve tadpoles of *Amaroucium pellucidum* all in the field at the same time were observed resting in contact with the surface film. They were at this time in a horizontal beam of light of 3000 m.c. A shadow was thrown upon these specimens by very rapidly passing the hand through the beam. Nine of the twelve immediately responded by becoming active, but all of them soon came to rest again in contact with the surface film. The lamp was now in the course of one-half minute moved from 40 cm. to 400 cm. from the organisms, reducing the intensity from 3000 m.c. to about 30 m.c. There was no response in any of the tadpoles. The hand was then slowly passed through the beam. One tadpole became active. Five minutes later this was repeated, but no response was obtained. The light in the beam was then intercepted for 20 hm.; again no response was obtained. The lamp was now moved so as again to increase the light to 3000 m.c. and the open hand with fingers spread passed very rapidly through the beam. Nine of the twelve specimens became active. Similar results were obtained in observations in front of a window provided with an opaque shutter. In reducing the illumination by closing the shutter activating responses could readily be induced by rapid movements through 10 to 15 cm., while slow movement produced no responses even when the shutter was entirely closed, resulting in total darkness excepting a weak ruby light which was practically neutral in its stimulating effect. (This light was commonly used throughout all of these experiments in making observations in low illuminations.) It is thus evident that the stimulating efficiency of reduction in illumination depends markedly upon the time-rate of reduction.

Amaroucium tadpoles, as previously stated, may be photopositive or photonegative. In *Euglena* and various other similar organisms in which a reversion of this sort occurs it has been found that photopositive specimens respond in a certain

definite way to a decrease in light, while they give no response to an increase, and that photonegative specimens respond in precisely the same way to an increase, but give no response to a decrease in light. In the tadpoles of *Amaroucium* there is, both in the positive and in the negative states, a definite response to a reduction and no such response to an increase in illumination. In other words, resting specimens become active when light is reduced, no matter whether they are positive or negative, but not when it is increased. There is, however, a difference in the character of the response in these two states. If the specimens are photonegative the first stroke or the first series of strokes of the tail is always toward the ocular side; if they are photopositive it is always toward the abocular side. The former was fully established in both species, the latter in *pellucidum* only. In *constellatum* the photopositive period is so short that the precise nature of the response could not be ascertained with certainty.

The difference in the activating response of the tadpoles in the positive and the negative states was particularly striking in one individual studied on August 27th in a vaseline enclosure under a cover-glass. This individual, a specimen of *Amaroucium pellucidum*, was mounted immediately after it had left the colony. It was strongly positive on the slide under the microscope and swam directly toward the window in front of which the observations were made. But when it reached the limit of the enclosure the tail became entangled in vaseline to such an extent that it was held fast. The body, however, could freely move back and forth laterally. After the tadpole came to rest the light was reduced by passing the hand in front of it. It immediately became active and it could be very distinctly seen that the first movement of the body was toward the abocular side (fig. 3). This was repeated many times with the same results. The light was also increased in various ways, but in no case was any reaction obtained, provided the specimen was at rest when the increase occurred. Ten minutes later the specimen was again shaded in precisely the same way in which it had been earlier, and now it responded by turning

toward the ocular side, in place of the abocular side, as it did before when it was positive. These results were repeatedly and consistently obtained, but in no instance did the activating response follow increase in illumination. Similar results

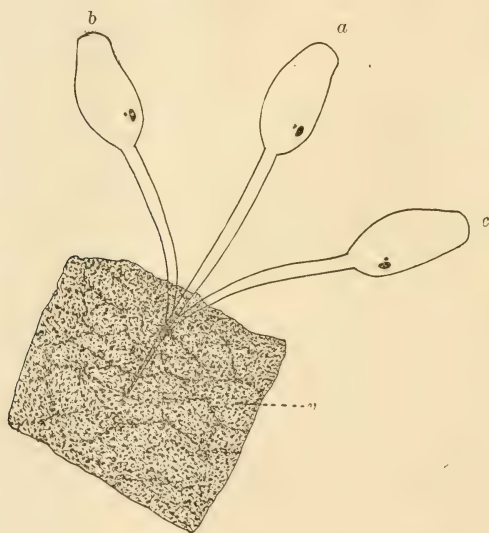


Fig. 3 Outline of *Amaroucium pellucidum* tadpole with tail embedded in vaseline. *a*, resting position; *b*, position assumed in reaction to shadow by specimen in photopositive state; *c*, position assumed in reaction to shadow by same specimen in the photonegative state. *v*, vaseline. Note that the tail bends toward the ocular side when the tadpoles are negative and toward the abocular side when they are positive.

were obtained in many other observations under various conditions. There is consequently no doubt but that in the tadpoles of *Amaroucium* both positive and negative specimens respond to reduction in luminous intensity by becoming active, that the response in the two states differs in character, and that they do not become active in either state when the intensity

is increased. In *Euglena* and other similar forms, as is well known positive specimens respond in a specific way to a decrease, while negative specimens respond in precisely the same way to an increase in light.

Let us now consider the question as to the cause of the direction of movement of the tail when the tadpoles become active in response to shadows. Why does the tail strike toward the ocular side when the tadpoles are negative and toward the abocular side when they are positive? Is the direction of the stroke of the tail dependent upon the localization of the stimulus?

As previously stated, the tadpoles of *Amaroucium* have alternate periods of rest and activity which are not associated with any observable changes in the environment. These periods are therefore probably related to changes in internal processes. Numerous observations were made on both species in attempting to ascertain precisely the nature of the response as they become active. These observations were unfortunately all made on negative specimens. It was found in these specimens that the first stroke of the tail is invariably toward the ocular side, provided they have been at rest fifteen seconds or longer. If they have been at rest for a shorter period of time, the stroke of the tail may be in either direction depending upon the direction of the last stroke preceding the rest period. If this stroke is toward the ocular side, then the first stroke in the active period will be toward the abocular side and vice versa. Now this is essentially what was found in the response of negative specimens to shadows. That is, when the tadpoles become active in response to reduction in illumination they act just as they do when they become active in the absence of any change in the environment. However, when a tadpole becomes active without any change in the environment there can be nothing in the nature of what is ordinarily understood by localization of the stimulus. If the direction of the stroke of the tail in these reactions is not related to the localization of the stimulus, it must be determined by the structure and physiological states of the organism. It would consequently appear that; since the action of the tail in response to shadows is like the action

without any change in the environment and since the direction of the stroke of the tail under the latter condition is determined by the structure and physiological states of the organism, it may be determined likewise under the former conditions. If it is thus determined, the response to shadows is purely an activating response, a response which is entirely independent of the localization of the stimulus. However this may be, evidence presented in the following pages shows that the tail in active specimens bends toward the ocular side when the light is reduced, indicating that reduction in illumination may be more than merely an activating stimulus.

Regarding the difference in the reaction to shadows in negative and in positive specimens, all that can be said with certainty at present is that in positive specimens the structure and physiological states and processes of the tadpoles are such that when the light is reduced the muscles contract in such a way as to cause the tail to bend toward the abocular side, while in negative specimens they are such that the same change in illumination causes them to contract in such a way as to make the tail strike in the opposite direction.

b. Active specimens

In the preceding section it was demonstrated that when the light is suddenly reduced resting tadpoles of *Amaroucium* immediately become active and that the activity may consist of a series of one or more strokes of the tail resulting in locomotion. In this section we shall consider the reactions in active specimens.

Numerous observations were made on the effect of suddenly reducing the illumination on specimens in which the tail was rapidly vibrating, and it was found that while there is no apparent change in the rate of vibration, the tail bends toward one side so as to vibrate in a new plane. In negative specimens it bends toward the ocular side, in positive ones toward the abocular side. This is particularly evident in specimens which are attached or held under the cover-glass.

If a number of tadpoles are put into a vaseline enclosure under a cover-glass some usually become entangled in the vaseline at

the edge of the enclosure. Others can be loosely held if the cover-glass is carefully pressed down. In some of the tadpoles in which locomotion is thus prevented it is usually found that the tail is rapidly vibrating. If now the light is suddenly reduced, the tail at once bends toward the ocular side and continues to vibrate (fig. 4). In the specimens which are loosely held this bending of the tail results in turning of the body toward the eye, producing a circular course, or if locomotion is prevented it

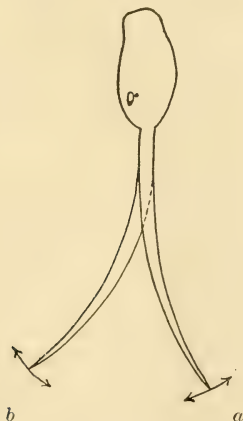


Fig. 4 Outline of tadpole of *Amaroucium pellucidum* representing reaction to decrease in illumination in active specimens. *a*, position of tail before stimulation; *b*, position of tail after stimulation; arrows, extent of vibration.

may result in the tail swinging toward the abocular side about the body as a pivot. In either case this movement continues only a very short time, after which the tail may assume its original position or it may bend in the opposite direction, then back again several times before it assumes its original position.

The time the tail holds the bent position after reduction of illumination was ascertained in a number of different specimens with a stop-watch. Eight to ten successive readings were made on each individual studied. The averages of the results

obtained vary from a trifle over one hundredth to nearly six hundredths of a minute. Thus it is evident that the reaction in question continues, at times, for only a very short period; indeed, so short that it resembles the reaction of skeletal muscles to a single induction shock. Under certain conditions, it is, however, in all probability the result of a series of impulses, for if the illumination is immediately increased again after it has been decreased, the tail always immediately bends in the opposite direction. Thus the reaction can be reduced to a very small fraction of a minute. It is consequently clear that the time the tail remains bent depends upon the condition of illumination. If the illumination is reduced and held there the tail remains bent longer than it does if the illumination is immediately increased again. Increase in illumination, therefore, inhibits the bending effect of decrease in illumination.

The degree of bending of the tail in the response to shadows is (like the extent of the stroke of the tail) within wide ranges largely if not entirely independent of the extent of the reduction in illumination. This was clearly established in numerous observations made in the same way as the observations on the extent of the stroke of the tail previously described.

The amount of reduction in light energy required to induce the bending reaction of the tail was not accurately ascertained, but it is certainly relatively small. Judging from the results obtained in numerous observations, the reduction necessary is about the same as that required to induce the activating response previously described; and it clearly depends upon the time-rate of reduction, just as in the case of the activating response, for it does not follow if the reduction is sufficiently slow, no matter how extensive it may be.

REACTIONS TO INCREASE IN ILLUMINATION

If the illumination is increased, resting *Amaroucium* tadpoles, as previously stated, remain quiet. There is no response even if intense direct sunlight is flashed upon them. But if they are active the tail at once bends toward the abocular side. Is this bending due merely to the inhibition of the effect of previous reduction in illumination or is it a response to a stimulus?

In tadpoles that are at rest the tail is usually bent considerably toward the abocular side. The extent of this bending varies with age. In very young tadpoles it is hardly noticeable, but in older ones it is marked (fig. 1). Now the question arises as to whether the tail, in bending toward the abocular side after the illumination has been increased, returns merely to the resting position or whether it goes beyond this position. The results obtained in numerous observations clearly show that it does go beyond this position, and they show, moreover, that, after the bending effect of decrease in illumination has ceased entirely and the tail is vibrating in the resting position, increase in illumination will still cause it to bend toward the abocular side. These facts seem to prove that increase in illumination does not merely inhibit the effect of a decrease in illumination, but acts also as a stimulus.

The observations referred to above were made on specimens of both species studied, some attached, others free. There was no observable difference in the reactions of the two species. Since these observations have a bearing on a number of problems aside from that stated above, I shall present in some detail the results obtained in three individuals, all in vaseline enclosures under cover-glasses; one with the anterior end firmly attached to the vaseline, the others free but limited in their movements.

In the attached specimen studies, *Amaroucium pellucidum*, there were alternate periods of rest and rapid vibration in the tail. When at rest the tail was very nearly straight, the curvature in it being unusually small (fig. 5, a). When the light was reduced by bringing the hand down in front of the stage, the tail immediately turned toward the ocular side and vibrated rapidly (fig. 5, b). This position was, however, held only a short time, 2 to 6 hm. Then, vibrating continuously, it returned to the resting position and sometimes slightly beyond: remained here 2 to 6 hm., then turned toward the ocular side again, etc., until it came to rest. If the hand was removed so as to rapidly increase the illumination while the tail was at rest there was no response, it remained at rest; but if it was removed before the tail came to rest, i.e., while it was still vibrating, no matter in

which position, it immediately turned sharply toward the abocular side (fig. 5, c), remained here a few moments, vibrating continuously, and then turned toward the resting position, sometimes reaching it, remained here a moment, after which it again turned toward the abocular side. Thus it continued to swing back and forth, but usually only a short time.

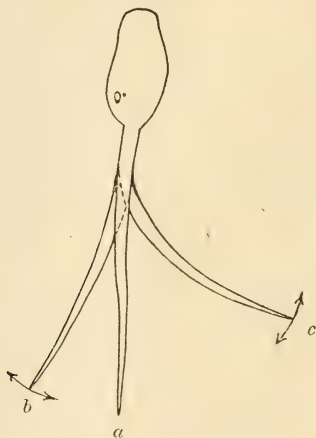


Fig. 5 Outline of *Amaroucium pellucidum* tadpole representing reaction to increase in illumination. *a*, resting position of tail; *b*, position assumed after decrease in illumination; *c*, position assumed after increase in illumination; arrows, extent of vibration.

Neither the position assumed in the shadow nor that assumed in the strong light was held for any considerable length of time if the luminous intensity continued without change. No record was made of the time it was held in this specimen, but in others it was found to vary from 1 hm. to 10 hm. It could be fairly clearly seen, however, that it was usually held considerably longer after increase than it was after decrease in illumination. Three series of ten readings each were made in reference to this period, two after reduction and one after increase in illumination. The averages of the results obtained in these series are,

respectively, 3.03 hm., 1.04 hm., and 6.74 hm. These readings were unfortunately all made on different individuals, so that the results obtained are not so conclusive as they would be if they had all been made on the same individual. They strongly support, however, the conclusion reached after numerous casual observations, i.e., that the position of the tail is held considerably longer after increase than it is after decrease in illumination.

Under either condition, however, the bent position of the tail is held longer when the illumination which induced it is continued than it is when this is not continued. That is, if the reduction in light is immediately followed by an increase, then the tail, after bending toward the ocular side, immediately bends toward the abocular side and vice versa. Thus by moving the hand in front of the microscope so as to change the luminous intensity, the tail can be made to swing back and forth in harmony with the hand as rapidly as it can be raised and lowered. A number of readings were taken in reference to this rate, and it was found to average 20 complete oscillations per 8 hm. 250 per minute, or 0.4 hm. per oscillation. This would indicate that the tail under these conditions holds the bent position in either direction less than 0.2 hm.

These results bring out in a striking way the difference between the activating response and the bending response. The former occurs only in resting specimens. It is induced only by reduction in illumination and it is independent of the illumination which follows the reduction. The latter occurs in active specimens. It can be induced by increase as well as by decrease in illumination, and it depends in character upon the illumination which follows that by which it is induced.

Since the extent of the lateral movements of the tail toward the ocular side when the light is decreased and toward the abocular side when it is increased is greater than it is in the oscillations under constant illumination, and since the lateral positions of the tail are held much longer when the illumination which induced them is maintained than when it is changed, it seems evident that both decrease and increase in illumination must be considered as a stimulus; the former causing contraction in

the muscles on the ocular side and the latter on the abocular side of the tail.

The contraction caused by reduction in illumination as shown above continues only 1 to 3 hm. This is similar to the result obtained in a nerve-muscle preparation by a single induction shock. The contraction caused by an increase in illumination on the other hand may be so long that it is evidently more like the results obtained by a series of induction shocks. A reduction in light, therefore, probably produces in the eye of the tadpoles of *Amaroucium* a single impulse which passes to the muscles of the tail causing a contraction of short duration in the muscles on the ocular side, while an increase in light produces a series of impulses which passes to the muscles of the abocular side causing a contraction much longer in duration; in fact a contraction similar to a tetanus.

These conclusions are supported in every detail by the results obtained in the observations on the two free specimens mentioned above, both belonging to the species *pellucidum*. One was loosely held under the cover-glass; the other was held in an indentation in the vaseline at the edge of the enclosure under the cover-glass in such a way, that while it could not progress, both the anterior and the posterior end of the body could move back and forth laterally. We shall consider the latter specimen first. This one was observed for about five minutes after it was mounted, before it was subjected to changes in illumination, and it was found that during this entire time the tail vibrated continuously and turned alternately quite regularly to the right and to the left, resulting in a change in the position of the body (fig. 6, *A*, *B*). The preparation was now shaded by passing the hand down in front of the stage and the tail at once turned toward the ocular side. This forced the posterior end of the body in the opposite direction against the edge of the indentation in the vaseline. Here it remained. The tail, however, continued to vibrate and it turned from side to side at intervals of approximately 2 hm. The hand was now raised; the tail immediately turned sharply toward the abocular side forcing the posterior end of the body toward the ocular side against the

opposite edge of the indentation (fig. 6, *B*). Here the tail continued to vibrate, turning fairly regularly from side to side, usually through an angle of about 30° , but occasionally sufficiently to force the body toward the abocular side against the opposite edge of the indentation. These observations were continued until the specimen became attached and began to metamorphose.

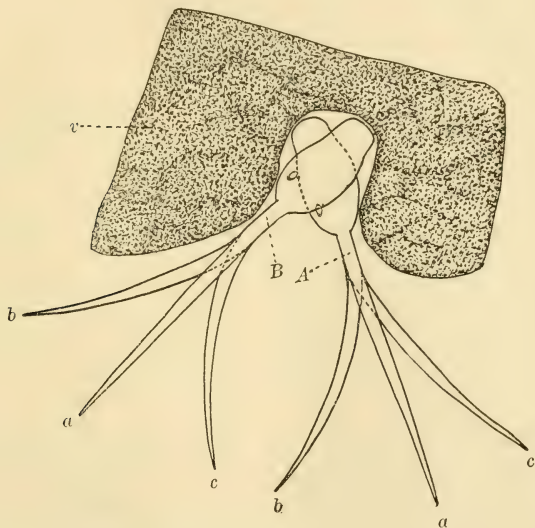


Fig. 6 Outline of *Amaroucium pellucidum* tadpole representing reaction to increase in illumination. *A*, position assumed after decrease in illumination; *B*, position assumed after increase in illumination; *a*, *b*, *c*, different positions of tail assumed without change in illumination; *v*, vaseline.

The results obtained throughout were in harmony with those presented above.

Similar results were also obtained in the observations on the specimen loosely held by the cover-glass. This specimen was so held that it could not progress, but could swing laterally in the plane of the slide in either direction around the central portion

of the body as an axis (fig. 7). Whenever the light was suddenly reduced by passing the hand down in front of the microscope while the specimen was at rest, it immediately became active, the tail

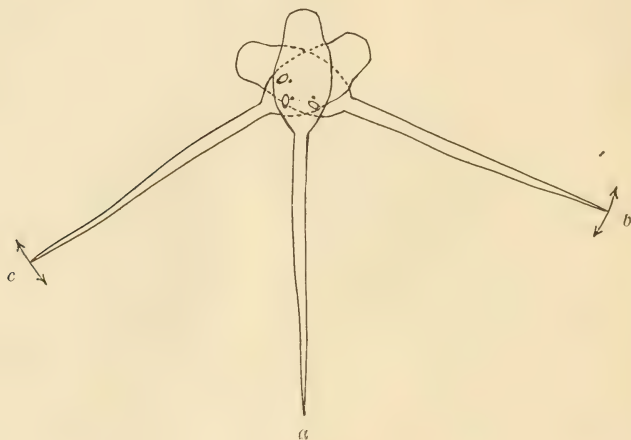


Fig. 7 Representing reaction to increase in illumination in *Amaroecium pellucidum* tadpole loosely held under cover-glass. *a*, resting position; *b*, position assumed after reduction in illumination. Note that the tail is practically straight but that it vibrates as indicated by the arrows. If the illumination is now increased, the tail bends toward the abocular side and the tadpole swings into position *c*, where the tail again becomes straight and vibrates as indicated by the arrows. If the illumination is now decreased the tadpole returns to position *b*, or nearly to this position. It usually swings somewhat further after the light is increased than it does after it is decreased. The fact that the tadpole, in response to an increase in illumination, swings from position *b*, in which the tail is practically straight, to position *c*, shows that the increase in illumination does not merely inhibit the effect of a preceding decrease, but acts as a stimulus causing the tail to bend toward the abocular side.

turning toward the ocular side and vibrating rapidly. This caused the posterior end of the body to swing in the opposite direction. (fig. 7, *b*). Thus it continued to swing usually 90

to 180° , but sometimes 360° or more. Then the tail became straight and the swinging stopped, but the vibration usually continued. If, now, the hand was raised so as to increase the illumination, the tail immediately turned toward the abocular side and the posterior end of the body began to swing toward the ocular side. In this direction it continued to swing usually considerably farther than it did in the opposite direction after shading. If the hand was removed before the body stopped swinging toward the ocular side in response to a shadow, the direction of swinging was immediately reversed. If it was not removed until after the vibration of the tail as well as the lateral swinging had stopped, there was no response. These experimental observations were repeated many times on this individual and a considerable number of times on another with the same results.

It is well known that if one of a pair of antagonistic muscles relaxes after having been contracted for some time, the opposing muscle becomes shorter than it is in the resting condition. May not then the bending of the tail toward the abocular side on increase in illumination be due to the previous bending in the opposite direction and not to the action of light as a stimulus? In the reactions of the first two individuals described above, I was never quite certain that this possibility was excluded, but in those of the third individual it has been excluded, for in this individual it was clearly seen that the bending reaction of the tail toward the abocular side in response to increase in light could be induced when the tail was straight and the antagonistic muscle relaxed, quite as readily as it could when the tail was bent toward the ocular side and this muscle contracted. The results obtained in this individual, therefore, seem to prove conclusively that, while an increase in the illumination of the field inhibits the effect of a decrease, it acts also as a stimulus.

The effect of increase in illumination depends upon the time-rate of change of illumination just as does the effect of decrease in illumination. If the light is gradually increased, there is no response, no matter how extensive the increase may be.

The magnitude of the response to increase in illumination is, like the response to decrease in illumination, within wide ranges

largely if not entirely independent of the amount of change in illumination. Numerous observations were made on the extent of the bending in the tail in response to direct sunlight flashed on individuals in weak light in comparison with the extent of the bending in the same individuals in response to diffuse sunlight. The extent of bending could be fairly accurately ascertained by noting the relation between the tail in various positions and stationary particles of debris found in the solution. While there was considerable variation in the magnitude of the reaction, no consistent difference under the two conditions of illumination was observed. Like results were obtained in observation on the effect of light much lower than diffuse sunlight. The response to increase in illumination is consequently of the 'all-or-none' type.

Increase in illumination is not an activating stimulus. It does not cause quiet specimens to become active and, so far as the evidence at hand indicates, it does not cause active specimens to become more active. The tail apparently vibrates as rapidly in the weakest light (white or red) in which it can be seen as it does in direct sunlight.

In conclusion, then, it may be said that increase in illumination probably has little or no effect on the rate of movement in *Amaroucium* tadpoles,⁴ that it inhibits the effect of decrease in illumination in reference to direction of movement but not in reference to rate of movement, and that it acts as a stimulus causing increase in bending of the tail toward the abocular side.

FUNCTION OF THE EYE

If a number of tadpoles are resting on a horizontal glass surface, it is found that in some the eye is so situated that light from directly below enters the opening of the pigmented cup and illuminates the ends of the nerves on the inner surface of the cup, and that in others it is so situated that light from below strikes the outside of the opaque cup and consequently does not reach the nerve endings on the inside (fig. 1). Grave maintains⁵

⁴ All that this statement implies is that if the period of exposure is short, the intensity of the light has little or no effect on the activity of the tadpoles of *Amaroucium*. This is probably not true if the period of exposure is long.

⁵ Personal communication.

that if the light from the mirror is cut off while tadpoles on a slide are observed under a microscope, it can be seen that only those become active in which the opening of the eye-cup faces downward. This would indicate that the activating reactions to reduction of light are due to a decrease in the illumination of the nerve endings in the eye.

I repeated these experiments, but was unable to obtain conclusive results. By means of other methods, however, I obtained results which confirm Grave's conclusion and demonstrate that in addition to the activating reactions observed by Grave the bending or directive reactions of the tail, which in the preceding experiments were induced by changes in the illumination of the field are also dependent upon impulses received through the eye. In other words, it was found that all of the reactions described in the preceding pages can be induced by changes in the illumination of the retina without any change in the luminous intensity of the field, or even with changes in the wrong direction, showing clearly that the eye functions in all of these reactions as a light receptor.

In these observations two methods were employed. One consisted in studying the effect in a horizontal beam of light of changes in the axial position of free-swimming specimens such that different surfaces of the eye become exposed; the other consisted in noting the reactions in attached specimens when the source of light was changed in position so as to illuminate different surfaces of the eye. In both it was possible to ascertain the effect of changes in the illumination of the retina.

First method. If a number of specimens are put into a vaseline enclosure under a cover-glass, some usually become attached to the vaseline and others get into narrow spaces between the cover and the slide so as to prevent locomotion. With such specimens under the binocular in the dark-room, a limited portion of any desired surface of the eye can readily be illuminated by changing above and below the stage, the position of a small electric bulb containing a concentrated filament. In this way, numerous observations were made on the effect of increasing and decreasing the illumination of the retina. The results

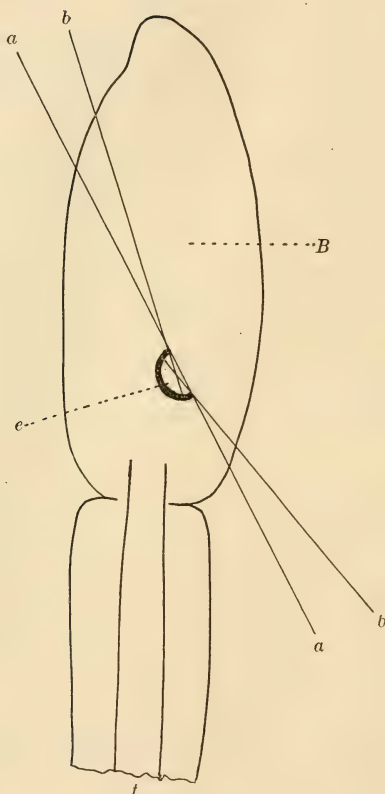


Fig. 8 Outline of the anterior end of tadpole of *Amaroucium*, showing the effect on the illumination of the retina of slight movements of the source of light. *b*, body; *t*, tail; *e*, section of pigmented eye-cup; *a*, *b*, different positions of source of light. Note that if the source of light is to the right of the line *a-a* the inner surface of the eye-cup which contains the optic nerve-endings is illuminated and that if it is to the left side of this line the inner surface is shaded. Note also that slight movements in either direction from the line *a-a* may produce profound changes in the illumination on the inner surface of the eye-cup.

obtained were all consistent. Without going into details, they may be stated categorically as follows: *a*) If the lamp is moved from a position in which the inner surface of the cup, the retina, is illuminated to one in which it is shaded (fig. 8), resting specimens immediately become active and the tail bends toward the ocular side. (Only negative specimens were studied.) This occurs even if the light is brought nearer so that the illumination of the field becomes stronger when the retina is shaded. *b*) If the lamp is moved from a position in which the retina is shaded to one in which it is illuminated there is no reaction in resting specimens, but in active specimens the tail turns toward the abocular side even if the lamp is moved away so as to decrease the intensity of the light in the field, provided it is not moved too far. *c*) If the lamp is moved back and forth so as to alternately illuminate and shade the retina, the tail swings back and forth in harmony with the lamp, even if the lamp is moved as rapidly as it can be with the hand.

It is surprising how little movement of the lamp is required in certain positions to induce these reactions. They were repeatedly obtained by movement back and forth over a distance of less than 2 cm.

Second method. The results obtained by the second method show that reactions similar to those described above are induced if the light remains stationary and the axial position of the tadpole changes.

If a number of tadpoles are put into a drop of water on a slide they tend to swim along the edge of the drop without rotating on the longitudinal axis. Thus given individuals frequently swim several times around the drop changing their axial position through 360° each time without rotating on the axis. During this process it is evident that if they are exposed in a horizontal beam, different surfaces of the eye become successively exposed to the light, so that in one position the retina is fully exposed, while in others it is shaded by the opaque cup of the eye (fig. 9). Now, it was found that when the retina becomes shaded as the tadpoles proceed along the edge of the drop, they suddenly turn sharply and very rapidly toward the ocular side,

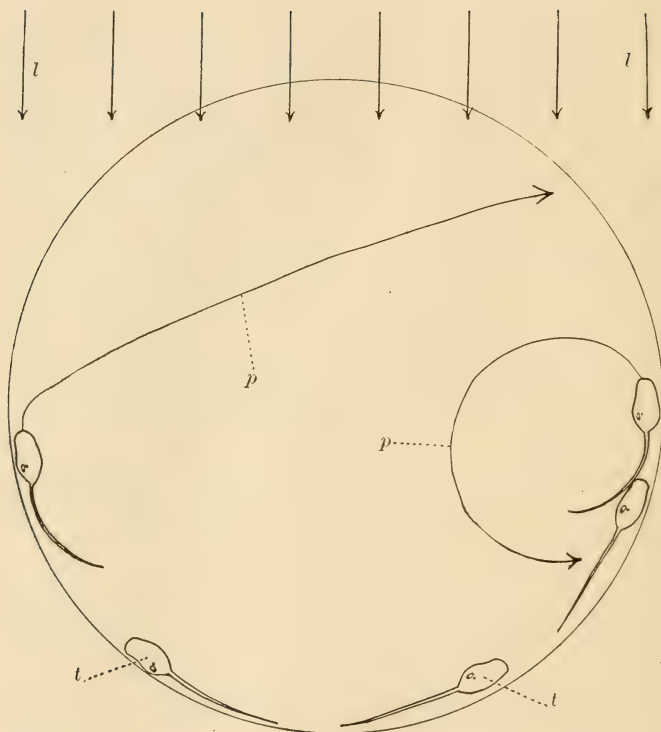


Fig. 9 Diagram representing the effect on the behavior of tadpoles of Amaraucium of sudden changes in the illumination of the retina. *t*, tadpoles swimming along the edge of a drop of water without rotating on the longitudinal axis; *l*, light rays; *p*, paths taken by tadpoles. Note that when the tadpole, in swimming along the edge of the drop, reaches a position in which the longitudinal axis is approximately parallel with the direction of the rays, it suddenly turns sharply and leaves the edge of the drop. In the position to the right this is owing to a decrease and in the position to the left to an increase in the illumination of the retina.

leave the edge of the drop like a flash, make a small circle and return again somewhat farther back. Then they proceed along the edge again and when the retina again becomes shaded they turn again. This may be repeated several times in succession, the reaction occurring each time approximately in the same location. Similar reactions occur when the retina becomes illuminated, but the turning is not nearly so sharp.

These results together with those presented above prove conclusively that the eye functions in all of the photic reactions observed.

The fact that changes in the position of the organism in relation to the source of light resulting in changes in the illumination in the eye may induce changes in the direction of locomotion is of great importance, for it gives an insight into the process of orientation, as will be shown presently.

It is remarkable that, while the eye in the tadpoles of *Amaroucium* is unquestionably useful, it functions, in some cases, for only a few minutes during the life-time of the individual, and its eyeless successors (zooids), which may live for several years producing, vegetatively, thousands of generations none of which have eyes but all of which may produce tadpoles with eyes fully formed and apparently perfect in function at birth. This seems to show that the origin of function in the eye of the individual is independent of experience, and that it must be in some way specifically associated with a substance (an eye determiner) which, without any apparent relation to function, is capable of passing from tadpole to tadpole through thousands of generations and millions of individual zooids.⁶

PHOTIC ORIENTATION

If *Amaroucium* tadpoles are exposed in a horizontal beam of light, they swim fairly directly toward or from the light, i.e., they orient fairly precisely. If the direction of the beam is changed, they immediately turn sharply toward or from the light until they are again oriented. What, now, are the factors

⁶Grave deals extensively with this problem.

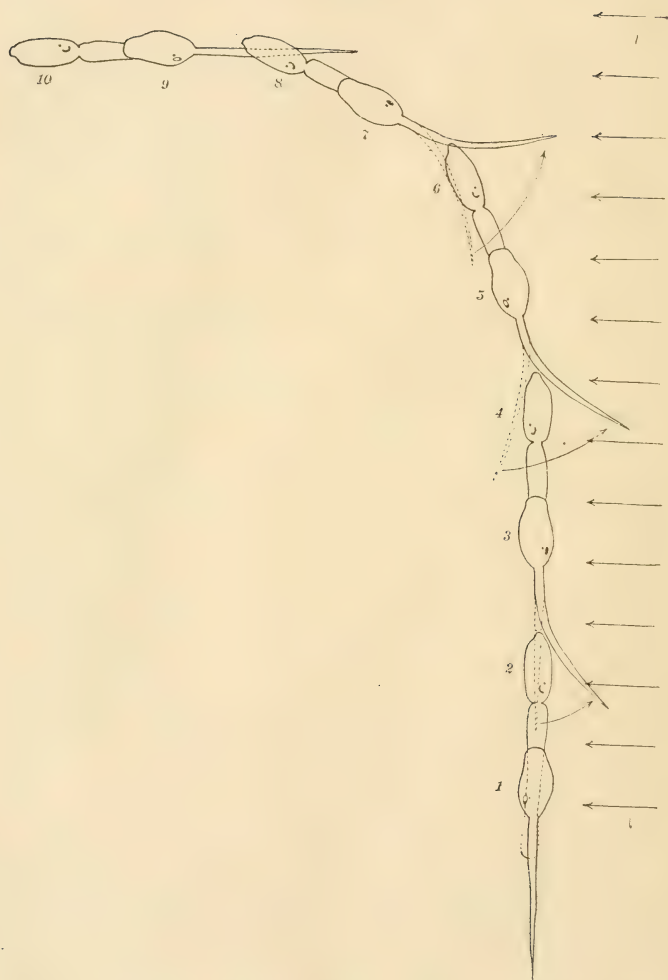


Fig. 10 Diagram representing the process of photic orientation in *Amarrucium* tadpoles. *l, l*, horizontal beam of light; 1 to 6, different positions of tadpoles. In position (2) the retina is fully exposed, but as the tadpole reaches position (3) it becomes shaded, owing to the rotation on the longitudinal axis. This causes the tail to bend toward the ocular side, which, owing to rapid rotations,

involved in the process of orientation and in the retention of the oriented axial position?

The movements of the tail of the tadpoles are normally so rapid that it is impossible to see directly how orientation is accomplished. These movements can readily be reduced in rate by means of lowering the temperature or by adding narcotics. But unfortunately it was found that when, under these conditions, they become slow enough so that they can be seen, orientation ceases. It was consequently not possible to ascertain directly what occurs during this process. Orientation can, however, be fairly satisfactorily explained by the application of the facts concerning photic responses presented in the foregoing pages.

We shall consider orientation in negative specimens first. In these it was found, 1) that a sudden decrease in the illumination of the nerve-endings on the inner surface of the pigmented cup of the eye induces the tail to bend toward the ocular side, resulting, in free swimming individuals, in a sharp turn of the anterior end toward this side; 2) that an increase in illumination induces, in active specimens, bending of the tail and sharp turning of the anterior end in the opposite direction; 3) that the former reaction may be induced by a change in the direction of the rays of light or by a change in the axial position of the body from one in which the inner surface to one in which the outer surface of the cup is directly exposed to the light; 4) that the latter reaction may be induced by a change precisely opposite in character, and 5) that these reactions alternate in harmony with alternate decrease and increase in illumination of the optic nerve-endings.

If a tadpole in a horizontal beam of light is so directed that the light strikes it at an angle of, e.g., 90° with the longitudinal axis, the inner and outer surfaces of the pigment cup of the eye are alternately turned toward the light, owing to the rotation

results in turning from the light. In position (5) the retina becomes fully illuminated. This causes bending of the tail toward the abocular side, which again, owing to the rapid rotation, results in turning from the light. Thus the tadpole continues to turn from the light each time the retina becomes shaded or illuminated until it is oriented and rotation on the longitudinal axis no longer produces sufficient changes in the illumination of the retina to induce reactions.

on the longitudinal axis (fig. 10), and this results in alternate decrease and increase in the illumination of the optic nerve-endings; when the inner surface of the cup faces the light the nerve-endings are fully exposed and when the outer surface faces it, they are shaded by the wall of the cup which is very nearly opaque. Whenever the illumination of the optic nerve-endings decreases the tail bends and the anterior end turns sharply toward the ocular side, and whenever it increases the tail bends in the opposite direction (fig. 10, 2). The eye is so situated that when the body rotates from a position in which the eye is on the shaded side to one in which it is on the illuminated side or a little beyond (fig. 10, 3) the inner surface of the eye-cup and the optic nerve-endings become shaded. The tail then immediately bends sharply toward the light and downward, but owing to the extremely rapid rotation of the body the anterior end is turned from the light (fig. 10, 5). But as the body rotates the eye is again carried to the shaded side, and as soon as it passes a little beyond, the inner surface of the cup becomes exposed to the light, the illumination of the optic nerve-endings increases and the tail responds by bending toward the abocular side, i.e., toward the light and somewhat downward. Owing to the rapid rotation, however, the anterior end is again turned from the light just as in the preceding half of the rotation (fig. 10, 7). Thus it continues until it is directed from the light and rotation on the longitudinal axis no longer produces changes in the illumination of the optic nerve-endings that exceed the threshold. In this direction it continues, not because it is continuously stimulated and held upon its course like a weather-vane in the wind, or like Hammond's 'mechanical dog,' as maintained by some (Loeb, '20, p. 68,) but because it tends to continue on a direct path in the absence of directive stimulation. And if, owing to obstruction or internal stimulation, it is thrown out of this course so that rotation again results in changes in illumination of the optic nerve-ending, the reactions just described are repeated and the organism again becomes oriented.

In positive specimens, as we have seen, the tail, in response to changes in the illumination of the optic nerve-endings, bends

in precisely the opposite direction from what it does in negative specimens. The anterior end is consequently turned toward the light in place of from it when, owing to rotation on the longitudinal axis, the optic nerve-endings are subjected to changes in illumination, and this turning continues until the organism proceeds directly toward the light and rotation on the longitudinal axis no longer results in changes in the illumination of the retina large enough to stimulate. It remains oriented for precisely the same reason that negative specimens remain oriented, i.e., because in the absence of directive stimulation it tends to continue in the direction in which it is going.

Photic orientation in the tadpoles of *Amaroucium* is consequently in principle essentially the same as it is in *Euglena*, *Stentor*, and numerous other organisms. It is dependent upon shock reactions of the 'all-or-none' or 'trigger' type. And the eye functions in the process of orientation much like the eye-spot in *Euglena* (Mast, '11) or the eye in *Planaria* (Taliaferro, '20).

Crozier ('18, p. 491) maintains that in *Chiton* orientation in light cannot be dependent upon reactions to changes of luminous intensity, because it responds to decrease in illumination when it is negative as well as when it is positive. He says: "It may be emphasized here, as in the case of certain pedate holothurians. . . . that the simultaneous presence of *photonegative* orientation and a precise *negative* response to shading, without any response (of the part concerned in orienting reactions) to increased illumination, is thoroughly inconsistent with the idea that photonegative orientation is brought about by a stimulation induced through any change in light intensity, as such." This conclusion may be correct, but the evidence presented in support of it (stated above) is not very strong: for, while we have in the tadpoles of *Amaroucium* precisely what Crozier maintains for *Chiton*, namely, reactions to shadows in negative as well as in positive specimens, orientation in these creatures is clearly dependent upon changes of luminous intensity.

WAVE LENGTH AND STIMULATION

The effect of wave-length on stimulation was only superficially investigated, but the results obtained indicate clearly that the longer waves of light have a much higher stimulating efficiency in the tadpoles of *Amaroucium* than they do in *Volvox*, *Pandorina*, and other similar organisms.

The tadpoles of *Amaroucium constellatum* were found to orient fairly precisely in light from a 25-watt tungsten ruby lamp at 100 cm. distance passed through two plates of ruby glass and a fairly heavy sheet of tissue paper. In *Volvox* and *Pandorina*, on the other hand, no responses whatever were observed in light direct from the same lamp without any obstruction even at 5 cm. distance. Since these are quite as sensitive as the tadpoles to white light, it is evident that the tadpoles are much more sensitive to red than *Volvox* and *Pandorina*.

DISCUSSION

Among the various factors functional in the process of orientation the character of the orienting response and its relation to the stimulating agent is one concerning which there is at present much contention.

Is orientation due to a series of shocks or trigger reactions of the 'all-or-none' type dependent upon the time-rate of change in illumination and bearing, only in so far as the threshold is concerned, a definite quantitative relation to the energy received; or is it due to reactions which themselves bear a definite quantitative relation to the energy received, reactions, which are not dependent upon the time-rate of change in illumination and in which there is nothing in the nature of a threshold? This sort of reaction has been referred to as a reaction to constant stimulation. It seems to hold within a limited range in some of the plant structures, e. g., certain plumules (Arisz, '11 and '15), and I am inclined to think that it may hold for *Eudendrium* (Mast, '11, p. 163; Loeb and Ewald, '14; Loeb and Wasteney, '17) and for other sessile forms in which orientation is irreversibly fixed, but I hold that the evidence presented by Loeb, Garrey, and others in favor of this view in so far as it concerns other animals is inconclusive.

Garrey ('17, '18) maintains that in insects orientation is due to difference in the tonus of like muscles in the legs on opposite sides of the body. He holds, if I understand him correctly, that the degree of tonus bears a specific quantitative relation to the amount of light received by the eyes, such that if one eye received more light than the other the tonus of the muscles in the legs on one side will be proportionally greater or less than that of like muscles in the legs on the opposite side, that difference in tonus, by inducing difference in flexure, results in difference in the length of the steps on opposite sides, thus causing the body to turn until both eyes are equally illuminated and the animal is oriented. He, consequently, maintains that, in the process of orientation, light acts continuously in proportion to the amount received by the photoreceptors.

Certain facts recently discovered by Dolley ('20, '21) and others discovered by myself ('20, not yet in press) seem to prove conclusively, that light in the process of orientation in insects (mourning-cloak butterfly, tachina fly, drone fly, robber fly) does not act continuously; and that while the effect of light on the tonus of the muscles may under certain conditions be a factor, it is normally of little or no importance, for certain insects turn toward the light even if the tonus of the muscles is such that its effect tends to make them turn in the opposite direction. This matter will be taken up in detail in a subsequent paper. I should, however, like to add here that it is difficult to see how, in a reversible system like a muscle, there could be a continuous action of the stimulating agent such as to produce a specific proportional relation in magnitude between the energy received and the reaction produced, for in periodic reversible systems like muscles, action in one direction cannot continue indefinitely. There must be time for restitution. All parts of a muscle cannot work in one direction all of the time. Either some parts must be at work while others are at rest or there must be periods of rest in the whole muscle, periods during which the stimulus has no effect, refractory periods. And, if this is true, the magnitude of the reaction in relation to the energy received from the stimulating agent depends upon the rate at which the energy is re-

ceived; i.e., in case of light, it depends upon the intensity of the illumination.

Shock reactions unquestionably function in the process of orientation in many of the lower forms, e.g., *Euglena*, *Stentor*, *Volvox*, blow-fly larvae, etc. (Mast, '11). Bancroft ('13), in a valuable paper on *Euglena*, agrees with this contention, but he also maintains that there are responses dependent upon the continuous action of light, which function in the process of orientation. I have elsewhere ('14) presented evidence indicating that Bancroft's conclusion in reference to the continuous action of light is questionable. However this may be, the orienting reactions in the tadpoles of *Amaroucium* are largely (probably entirely) of the shock, trigger, or 'all-or-none' type, those induced by an increase as well as those induced by a decrease in the illumination of the retina. They are dependent upon the time-rate of change in illumination and there is clearly a threshold. If this is exceeded, there is a reaction. Those reactions which are induced by decrease in illumination are of a given magnitude which is not increased no matter how great the reduction in illumination beyond the threshold may be. And this probably holds also for those which are induced by increase in illumination, although in these the magnitude of the reaction may possibly depend somewhat upon the magnitude of the stimulus. If there is actually such a relation it can, however, readily be accounted for by the assumption that more muscle fibers come into play when the stimulus is strong than when it is weak, just as is done in applying the 'all-or-none' law to cases in which such relations are known to obtain. This matter is of considerable importance and it should be more thoroughly investigated.

SUMMARY

1. The larvae of *Amaroucium* are much like amphibian tadpoles in shape. They have but one eye, which is located laterally at the posterior end of the body near the base of the tail. The eye contains a lens, a pigment cup and optic nerve-endings on the inner surface of the cup.

2. The reactions to light in the tadpoles of *Amaroucium constellatum* and *Amaroucium pellucidum* are essentially the same.

3. The tadpoles orient fairly precisely. When they emerge from the colonies they are strongly photopositive, but they remain positive only a few moments, after which they become photonegative and remain so until they become attached and begin to metamorphose.

4. In swimming the tadpoles rotate rapidly and continuously on the longitudinal axis, counter-clockwise as seen from the rear. Locomotion is produced by means of lateral strokes of the tail, rotation on the axis, probably by twisting of the tail during the lateral strokes.

5. They have alternate periods of rest and activity. If the light is rapidly reduced the resting specimens, both positive and negative, respond by becoming active, and the active specimens respond by changing their direction of locomotion, the positive specimens turning toward the abocular and the negative ones toward the ocular side.

6. Increase in illumination has no effect on resting specimens regardless as to how rapid or extensive it may be, but active specimens respond by changing the direction of motion. This was ascertained with certainty only in negative specimens. These turn toward the abocular side.

7. The reaction time is so short that if the hand is moved up and down in front of the microscope as rapidly as possible, alternately increasing and decreasing the luminous intensity, the tail in attached specimens swings from side to side in harmony with the movement of the hand.

8. If the illumination is gradually changed in either direction there is no response regardless as to how extensive the change may be. The responses to light are dependent upon the time-

rate of change in illumination. They are in this respect like the responses in *Euglena* known as 'shock reactions'—'Schreckbewegung.'

9. All of the photic reactions are probably due to changes in the illumination on the nerve-endings at the inner surface of the pigment cup of the eye. Movements of the light or of the tadpole that produce changes in illumination of the optic nerve-endings owing to the shadow cast by the pigment cup, are followed by the same reactions as like changes in the illumination of the entire field.

10. Orientation is the result of one or more shock reactions caused by the alternate shading and illumination of the optic nerve-endings, owing to rotation on the longitudinal axis. In negative specimens the former causes the tail to bend toward the ocular, the latter toward the abocular side; in positive specimens precisely the opposite. The one set of responses directs the organism from, the other towards the source of light. After the tadpoles are oriented, the retina is continuously approximately equally illuminated, the shock reactions cease, and they continue on the course established. They remain oriented not because they are held on their course by continuous stimulation, as is so frequently maintained, but because they tend to continue on a direct course when they are not stimulated.

11. Orientation in this organism is in no way dependent upon a balanced effect of stimuli acting continuously on symmetrically located photoreceptors in accord with the DeCandolle-Verworn theory of orientation accepted by Loeb and others.

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Resumen por el autor, Arthur Calvin Walton.

La espermatogénesis de *Ascaris felis* Goeze.

Los principales resultados de este estudio son los siguientes: 1) *Ascaris felis* Goeze presenta nueve cromosomas en la condición haploide, ocho autosomas en tetradas y un heterocromosoma en hexada. 2) Este heterocromosoma está formado por un idiosoma fusionado con el extremo de un autosoma. 3) Esta unión tiene lugar en el espermatozoido de primer orden, y el idiosoma es una entidad cromática independiente durante el periodo de desarrollo de las espermatogonias. 4) El idiosoma es del tipo X. y sufre reducción cuantitativa durante la formación de las espermátidas.

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THE SPERMATOGENESIS OF ASCARIS FELIS GOEZE

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TWO PLATES (TWELVE FIGURES)

INTRODUCTION

The work in this paper, on the spermatogenesis of *Ascaris felis* Goeze, the common *Ascaris* of the cat, is part of a broader work on the Ascaridae of the Canidae and Felidae. As shown in earlier papers (Walton, '16 a, '18), there can remain no doubt as to the separate morphological and cytological entities of the common ascarids of the dogs and of the cats. The work of Glaue ('08, '09), Edwards ('11), and Boveri ('11) was substantiated, while the work of Marcus ('06), Schöppler and Krüger ('12), and a number of earlier writers was shown to have been inaccurate—at least as to the nomenclature of the forms studied. (For a full discussion of the literature see Walton, '18.)

In 1911 Edwards made a brief cytological study of *Ascaris felis* and established the fact that a sex-chromosome complex was present. This complex he interpreted as an XY-pair of idiosomes, in which the X member of the pair was twice as large as the Y. Boveri ('11) under whom Edwards made his investigations, interpreted the results in a somewhat different manner. By analogy to the conditions found in *A. megalocephala*, Boveri believed that the sex-chromosome complex of *A. felis* consisted of only one small member which was attached to the end of an autosome.

In the writer's earlier work, he noted several instances which seemed to support the idea advanced by Boveri rather than that advocated by Edwards. These instances led the writer to make a careful study of the *A. felis* material in hope of being able to establish more fully the nature of the idiosome complex,

and the results seem to show definitely the presence of an X-type idiosome attached to the end of an autosome.

MATERIALS AND METHODS

The material used was obtained from freshly killed cats. The worms were immediately removed from the intestine of the host, placed in normal salt solution and kept at body temperature until they could be killed. The posterior end of the worm was clipped off and the testes were at once stripped out on the glass plate and fixed. Hermann's fluid and Flemming's fluid (strong) were found to give the most satisfactory fixations. The sections of the testes were made 4μ to 8μ in thickness. In general the material was stained by a modified iron-alum-haematoxylin method; dilute Delafield's haematoxylin being used after the mordant in the place of $\frac{1}{2}$ per cent aqueous solution of haematoxylin. This method, as in earlier work (Walton, '16 a), gives a better contrast between the chromatin matter and the yolk granules than can be obtained by the usual iron-alum-haematoxylin method of Heidenhain. Orange G and Bordeaux red were used as counter-stains.

EXPERIMENTAL EVIDENCE

a. Spermatogonia

As is the case in *A. canis*, the spermatogonia of *A. felis* are very small and the nuclear material tends to clump to such a degree that but little evidence can be gained by their study as to the chromosomal number. The spindle and centrosomes are quite distinct, however. This 'mass' division was closely studied. Of 430 cells examined during spermatogonial metaphases, 271 of these showed the presence of both a large and a small clump of chromatin material passing to each pole of the mitotic figure. The smaller clump of chromatin was apparently one-tenth of the size of the larger mass. The smaller clump of chromatin varied considerably in position; sometimes lying close to the larger body, and even connected to it by chromatin strands, and again, lagging far behind, widely separated from the main mass as it progressed towards the poles of the spindle.

The examination of 1103 spermatogonial nuclei which were in the 'resting stage' showed 917 containing two clumps of chromatin, one about ten times as large as the other (fig. 1). That this smaller body was not a plasmosome was shown by the staining reactions, as it responded in every case exactly as did the larger mass of chromatin material.

The cytoplasm of the early spermatogonial cells shows a slightly reticulated structure that gradually changes to a granular texture as the spermatogonia become older. By the time the last spermatogonial divisions are completed the granules have coalesced, becoming larger and fewer in number and taking a heavy plasma stain. These granules behave in the same manner as they do in *A. megalocephala* and *A. canis* (Walton, '16 b) and ultimately form the nutritive yolk cap of the spermatozoön.

b. Maturation stages

1. *First spermatocytes.* The chromatin of the spermatocytes during their growth period appears in the form of two irregular masses situated peripherally in the nucleus, and similar in all respects to the chromatin bodies noted in the spermatogonial nuclei. The mass of the smaller body is about one-tenth that of the larger. A distinct plasmosome makes its appearance, but gradually disappears at the time of the differentiation of the chromosomes.

Near the end of the spermatocytic growth period the two chromatin masses become an integral part of a system of peripherally placed linin fibers (fig. 2). Soon after becoming a part of this peripheral network, the two chromatin bodies show a segregation of their contained chromatin material into discrete bodies embedded in a thick background of linin. The larger of the two centers of chromosome formation gives rise to nine of these small aggregations of chromatin, while the smaller center gives rise to only one aggregation (fig. 3). These minute chromatin clumps assume the definite form of chromosomes as the linin background disappears. The nine chromosomes are of the tetrad form, while the single chromosome is a diad.

An examination of 762 nuclei in the late prophase stage showed two in which there were nine tetrad chromosomes of like size and one small diad chromosome, the latter being identical with the chromosome formed from the smaller chromatin mass of the early spermatocytes (fig. 4a). The rest of the nuclei examined showed eight tetrad chromosomes of like size and one that was about one-third larger (fig. 4b). This chromosome was apparently a tetrad with one end longer than the other. Among these 760 nuclei exhibiting this condition, there were 78 with a more or less distinct constriction midway of the large chromosome, varying from a very slight superficial indentation to an almost complete separation of the elements.

From this evidence it seems probable that, as in the case of *A. megaloccephala* (Boveri, '09; Edwards, '10), there is a consistent attachment—generally an indistinguishable union—of the small chromosome to the end of an autosome. Such a composite chromosome has a hexad form in *A. felis*, the small chromosome being a diad and the autosome a tetrad in form. The method of development of this diad chromosome from a small separate chromatin mass is so completely analogous to the formation of the X-type idiosome complex of *A. canis* from a similar separate chromatic mass in the early spermatocytic nuclei, that the interpretation of this chromosome as an idiosome of the X-type seems consistent. Thus we find that early in the development of the spermatocytes the haploid number of chromosomes, nine, clearly defines itself; eight of these normally being tetrads and one a hexad (fig. 4b). The hexad is formed by the attachment of the idiosome to the end of one of the autosomes.

In the metaphase of the first spermatocytic division the chromosomes are arranged in the equatorial plate, with the hexad chromosome apparently more centrally located than any of the autosome tetrads (figs. 5a and 5b). The great variation in the position is probably due to the antagonistic action of the idiosome and autosome components of the hexad which prevented either a distinctly peripheral or a distinctly central location. In three cases in which the idiosome was found unattached to an autosome, it occupied a definitely central position (fig. 5c).

The anaphase of the first spermatocytic division shows that there is a slight lagging in the division of the hexad chromosome (fig. 6a) and that there is a distinct lagging in the progress of the larger portion of the hexad towards the pole of the spindle (fig. 6b). The two daughter plates resulting from such a division (figs. 7a and 7b) show that one received nine diad chromosomes while the other received eight diads and one larger tetrad chromosome. Two types of second spermatocytes are thus formed, one with nine autosomes and the other with the nine autosomes plus the attached idiosome. The lagging during the division of the hexad chromosomes was caused by the slow response of the idiosome component, thus holding back the division of the autosome member. The division is along the plane of the transverse constriction of the autosome member of the hexad chromosome and not at the point of union of the idiosome and the autosome. The division is therefore qualitative. The arrangement of the chromosomes in five cells in which the idiosome was found separate from the autosomes during the anaphase condition, support this interpretation, inasmuch as the nine autosomes had divided without any signs of lagging, and the idiosome passed undivided to one pole, but in each case lagging behind its accompanying autosomes.

2. *Second spermatocytes.* The second division of the spermatocytes containing nine diad autosomes (fig. 8a) is entirely regular and quantitative, each spermatid receiving one-half the amount of the chromatic material in the form of nine monad chromosomes (fig. 10a).

The second division of the spermatocytes containing eight diads and one tetrad chromosome (fig. 8b) was also a quantitative division in that each daughter plate received one-half the amount of chromatic material in the form of eight monad autosomes and one diad heterochromosome (autosome plus idiosome) (fig. 10b). This division shows the lagging reaction of the idiosome in that the tetrad heterochromosome always separates at one end (the autosome) before it does at the other (the idiosome) (fig. 9a). The diad heterochromosome resulting from such division always lags behind the autosomes in the passage towards the poles of the spindle (fig. 9b).

One case of an unattached idiosome was found, there being ten diad chromosomes in the equatorial plate. A similar nucleus in the anaphase condition showed the dividing idiosome lagging behind the nine autosomes (fig. 11).

The spermatids formed from both types of spermatocytes of the second order show a clumped mass of chromatin with the centrosome embedded in one side of this mass (fig. 12). There is so little difference in the amount of chromatic material in the spermatids arising by the division of the two types of second spermatocytes, that there is no appreciable difference in size.

DISCUSSION

The results of Edwards ('11), closely approximated by the earlier work of the present writer (Walton, '16 a), have been checked carefully in this later examination of *A. felis* material and have been found to be in the main faulty only because the interpretations were based on too small an amount of material. Edwards reported that polar views of the metaphase plates of the first spermatocyte division showed nine tetrad chromosomes, one of which was asymmetrical and larger than any of the remaining eight. This large tetrad was composed of two unequal parts, the larger component being as large as one of the ordinary tetrads. The smaller component was about half the size of the autosome tetrads. The first division was transverse, and separated the unequal components of the large tetrad, which was undoubtedly to be classed as a sex chromosome, either of the XY-type or as an X-chromosome attached to the end of an autosome tetrad. Edwards inclined towards the first interpretation. Boveri ('11), after a review of the evidence presented by Edwards, advocated the second interpretation.

In the first division one daughter plate received eight diad autosomes and the larger (X) component of the heterochromosome; the other plate received eight autosomes and the smaller (Y) component of the heterochromosome. The second spermatocyte division was equational and regular, each spermatid receiving nine chromosomes (eight plus either X or Y). The spermatid receiving the X-chromosomes showed eight

chromosomes of equal size and the larger X-type; the spermatid receiving the Y-chromosome showed nine chromosomes, all about equal in size. According to Boveri's interpretation, each spermatid received nine autosomes, and the spermatids having the eight small and the one large chromosomes really had an X-type chromosome attached to one of the autosomes, thus making it larger than the others. The other type of spermatid did not receive any sex chromosome, therefore the chromosomes were all of equal size.

The present work has been based on a large amount of material and, because of the occasional appearance of forms differing from the normal, has thrown additional light on the nature of the sex chromosome complex of *A. felis*, thus showing that the interpretation of Boveri was the more correct.

The haploid number of the chromosomes is typically nine, and the diploid undoubtedly would be eighteen, although potentially the numbers are ten and twenty, respectively. Such potential figures are probable because the independent initial development of the idiosome, with only a secondary union with an autosome, shows that there were, momentarily at least, ten chromosomes in the early spermatocytic stage. This secondary union gives the haploid number of nine chromosomes, of which one is larger than the other eight.

The behavior of the idiosome is that of an X-type chromosome rather than that of an XY-type for several reasons. Chromosomes of the XY-type divide along their plane of syndesis in the first division, and are each quantitatively divided in the second. Chromosomes of the X-type are divided quantitatively in one division, but are not involved in the other, although they may be attached to the end of an autosome (Boveri, '09, and Edwards, '10). The idiochromosome in *A. felis* is not involved in the first division, passing unchanged to the second spermatocyte. The plane of division passes through the transverse axis of the autosome component of the heterochromosome, as most clearly shown in the nuclei in which the idiosome failed to become attached to an autosome, or was only partially connected. In the former case the idiosome passed undivided to

one-half of the daughter cells, while all the other chromosomes underwent division.

The longitudinal plane of the second spermatocytic division passes through the idiosome, attached or free, and clearly points to the fact that this sex chromosome must be of the X-type.

This behavior is in complete accord with the known series of events occurring in *A. megalcephala* where the X- type of idiosome is attached to the end of one of the autosomes, and therefore it is only logical to apply the same interpretation to *A. felis* and call the sex chromosome one of the X-type, and similarly attached to the end of an autosome.

Wilson ('10) has shown that in many insects the idiochromosome unit appears as a pseudoplasmosome during the growth period of the spermatogonium, distinctly separate from the chromatin masses from which the autosomes appear. Wilson has also shown that the 'Y' component of a sex-chromosome complex probably has little if any part in sex determination, oftentimes not appearing at all in the germ cells of certain individuals of species which generally show its presence. Wilson also states that in certain insects the various individuals may have the same number of chromosomes, but that these chromosomes may not always be of homologous types, though by any but the most careful examination of large amounts of material the difference may not be detected. Such a possibility in the material of *A. felis* must not be overlooked, and perhaps may account for the difference of interpretation between Edwards and Boveri and between Edwards and the writer. The same phenomenon of variation probably also accounts for the divergences from the normal in the behavior of certain chromosomes in some of the germ cells.

SUMMARY

The following are the most important points brought out in this study:

1. *Ascaris felis* Goeze shows nine chromosomes for the haploid number, eight tetrad autosomes, and one hexad heterochromosome.

2. This heterochromosome consists of an idiosome attached to the end of an autosome.

3. This union occurs in the primary spermatocytes, the idiosome being a separate chromatic entity during the spermatogonial developmental period.

4. The idiosome is of the X-type, undergoing quantitative division at the time of the formation of the spermatids.

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EXPLANATION OF PLATES

All the figures were drawn with the aid of a camera lucida, the projection distance being 410 mm. Spencer 2-mm. oil-immersion objective and compensating ocular $\times 10$ were used. Magnification of 3,200 diameters.

The following abbreviation is used: *id*, idiosome, the chromosome in which it is a part of the chromatic body from which it is derived.

PLATE 1

EXPLANATION OF FIGURES

- 1 Spermatogonium showing two unequal chromatin bodies in the nucleus.
- 2 Prophase, first spermatocyte, showing two unequal chromatin bodies in the nucleus.
- 3 Prophase, first spermatocyte, showing formation of chromosomes and the isolated position of the idiosome.
- 4a Late prophase nucleus, first spermatocyte, showing nine tetrad autosomes and the isolated idiosome.
- 4b Late prophase, first spermatocyte, showing idiosome attached to the end of one of the nine autosomes.
- 5a Polar view of metaphase plate, first spermatocyte, showing idiosome attached to the end of one of the autosomes.
- 5b Lateral view of metaphase plate, first spermatocyte, showing idiosome attached to the end of one of the autosomes.
- 5c Polar view of metaphase plate, first spermatocyte, showing idiosome not attached to an autosome.
- 6a Early anaphase, first spermatocyte, showing lagging division of the heterochromosome.
- 6b Anaphase, first spermatocyte, showing lagging of the heterochromosome.

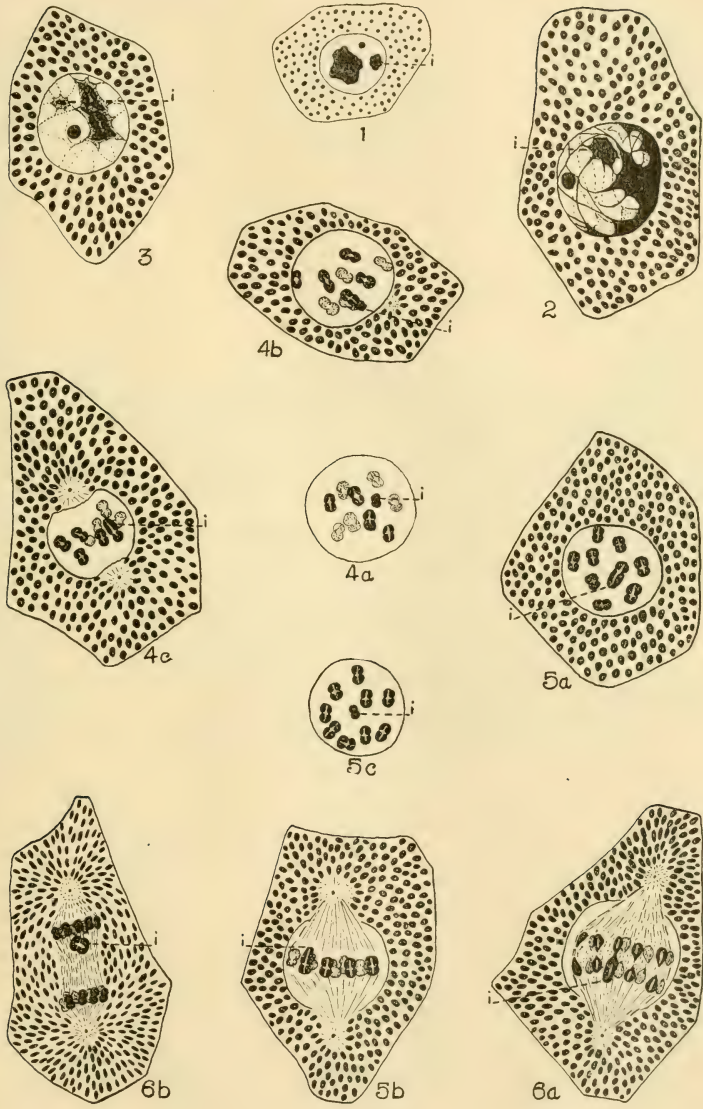
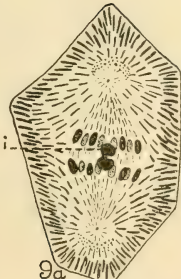
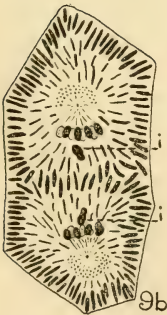
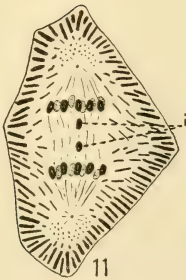
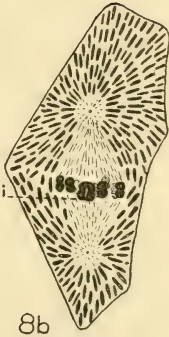
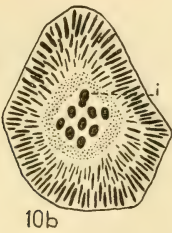
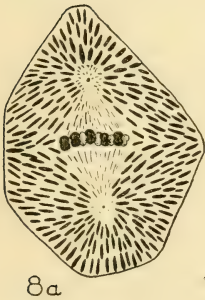
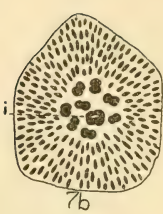
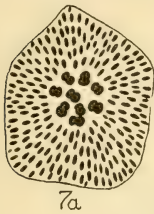


PLATE 2

EXPLANATION OF FIGURES

- 7a Anaphase plate, first spermatocyte, containing nine autosomes.
- 7b Anaphase plate, first spermatocyte, showing idiosome attached to the end of one of the autosomes.
- 8a Metaphase plate, second spermatocyte. Idiosome not present.
- 8b Metaphase plate, second spermatocyte. Idiosome attached to the end of one of the autosomes.
- 9a Early anaphase, second spermatocyte, showing lagging division of the heterochromosome.
- 9b Late anaphase, second spermatocyte, showing lagging of the heterochromosome.
- 10a Telophase, second spermatocyte. Idiosome not present.
- 10b Telophase, second spermatocyte. Idiosome attached to the end of one of the autosomes.
- 11 Late anaphase, second spermatocyte, showing idiosome free and lagging in division.
- 12 Spermatid, showing centrosome partially embedded in the nucleus.



Resumen por el autor, Charles Zeleny.

La dirección y frecuencia de la mutación en la serie de alelo-morfos múltiples de la variedad de ojo en forma de banda vertical de *Drosophila*.

1. En la serie alelomórfica de ojos en forma de banda vertical de *Drosophila melanogaster* se han observado tres genes definidos sin intermediarios, y todos los posibles cambios excepto el de completo á ultra-banda se conocen actualmente. 2. Las mutaciones reversivas son más frecuentes que las originarias, pero el origen reciente no es una explicación de la diferencia. 3. Las mutaciones intensas son tan frecuentes como las pequeñas. 4. La dirección de la selección, presencia ó ausencia de factores accesorios, y la dirección de origen no producen efecto sobre la dirección ó rapidez de las mutaciones. 5. No existen pruebas de la existencia de periodicidad. 6. Las mutaciones tienen lugar en los plasmas germinativos de los machos y las hembras y no están confinadas á un solo periodo de la historia de las células sexuales. 7. La frecuencia de la mutación en el locus de la variedad de ojos en forma de banda vertical es mayor que la de todos los factores accesorios combinados. 8. Los diferentes componentes de la serie mencionada son entidades definidas comparables á compuestos quimicos definidos ó estados fisicos. 9. No existe diferencia en la rapidez de la mutación desde á banda á ojo completo en los cultivos conservados á una temperatura de 20 á 22°C y los conservados á 27°C.

Translation by José F. Nonidez
Cornell Medical College, New York

THE DIRECTION AND FREQUENCY OF MUTATION IN THE BAR-EYE SERIES OF MULTIPLE ALLELOMORPHS OF DROSOPHILA¹

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FIVE FIGURES

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I. MATERIAL AND METHOD

1. Introduction

Series of multiple allelomorphs are valuable in studies of the nature of mutation, because the changes involve a single germinal material. In *Drosophila melanogaster* Meig the bar series, full to bar to ultra-bar, has an especially valuable combination of characteristics for such a study: 1) The details of the origin of the components are known; 2) the facet counts make possible a quantitative evaluation of the components; 3) the heterozygotes may be recognized; 4) the mutations are frequent enough to furnish an adequate measure of rate; 5) the change from bar to ultra-bar may be considered as an intensification of the first change from full to bar; 6) both direct and reverse mutations have been observed.

¹ Contribution from the Zoological Laboratory of the University of Illinois, no. 188.

2. The components of the bar series

The relative sizes and facet numbers of the different eyes are shown in figure 1. Full eye (*F*), bar (*B*), and ultra-bar (*U*) are sufficiently distinct for purposes of identification, and the same may be said of the heterozygotes. H_1 is the heterozygote containing one bar and one full factor, while H_2 has one ultra-bar

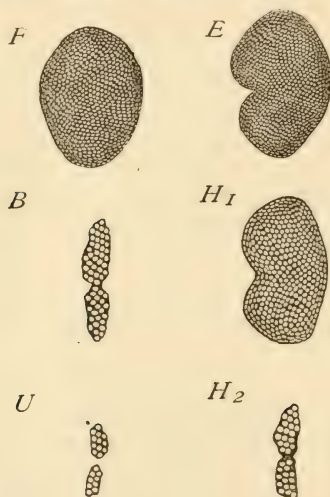


Fig. 1 Eyes of females of *Drosophila melanogaster* Meig. *F*, full eye as found in wild flies; *B*, bar eye in unselected stock; *U*, ultra-bar eye with typical separation into two parts; *E*, emarginate eye, produced by the bar factor plus accessory factors; H_1 , heterozygote of full x bar; H_2 , heterozygote of full x ultra-bar.

and one full factor. *E*, emarginate eye is not a member of this allelomorphic series, but is due to an accessory factor added to bar. Full-eye females at 27°C. have a mean facet number of 810.6, and at the same temperature unselected bar has 58.8 and ultra-bar 22 facets. The dominance likewise shows an intensification. Bar in crosses with full has a dominance coefficient of 0.23 and ultra-bar one of 0.85 when complete dominance is represented as 1.00 and complete recessiveness as 0.00 (Zeleny, '20).

3. *The origin of the series*

The original change, that from full to bar, was obtained in February, 1913, by Tice ('14) in a single male in an experiment involving rudimentary and long-winged flies with normal full eyes. No recurrence of the mutation has been mentioned by later observers.

The second change, that of bar to ultra-bar, also occurred in a single male (Zeleny, '20, p. 300). It was observed on October 20, 1917, in the second generation of selection for low facet number in a white bar stock obtained by crossing red bar with white full.

That the change from full to bar is reversible was demonstrated in this laboratory by May ('17), who obtained eleven mutations from bar to full during the period November 15, 1915, to June 7, 1916.

4. *Material for the present report*

The present report is based upon four sets of observations:²

1. General observations of full-eyed stocks were made over the period 1914 to 1920.

2. The selection series in white bar number 127 at 27°C. consists of forty-two generations of selection for low and for high facet number extending over the period September 26, 1917, to July 15, 1919, or approximately twenty-two months. During this time facet counts were made of all the individuals, and mutations were therefore located at once.

3. With the establishment of the ultra-bar stock, careful observations of all individuals, with facet counts at 27°C., were made over the period October 20, 1917, to July 23, 1918, or approximately nine months. As in the previous case, mutations could be accurately located.

4. The whole group of full, bar, and ultra-bar stocks was kept under observation for mutations during the period January to

² For excellent care of the living material and for continued help during the course of the investigation I am indebted to Dr. Joseph Krafka, Jr., who was my assistant from 1916 to 1919, and to Mr. D. H. Thompson, who has occupied the same position since that time.

October, 1920, or approximately nine months. During the last six months observations were made of all individuals in each generation. Facet counts were not made, but the examinations were sufficiently careful to insure the location of all mutations with the exception of heterozygotes between bar and ultra-bar.

5. Method

Efforts were concentrated upon the avoidance of contamination and the immediate recognition of mutants.

1. *Contamination.* May ('17) has discussed the reasons why contaminations may be excluded as an explanation of the reverse mutations of bar to full. The same argument applies to the different mutations described in the present paper. Special care was taken to avoid contamination, but the reader will be more interested in the evidence against such an explanation of the facts. As shown in table 2, a great variety of stocks was used, including a number with various mutant characters in addition to those involving eye facet number. In every case where an eye mutation occurred these other characters were those proper to the particular stock. Also, as in May's observations, the female mutants were all heterozygotes, while on the theory of contamination homozygotes should occasionally appear.

2. *Evidence for immediate recognition of mutations.* With the single exception already noted, the heterozygotes between the different members of the bar series are intermediate in character between the parents and sufficiently different from either of them, so that mutations in daughters as well as in sons may be recognized at once. In case the primary change occurs in one of the sex chromosomes of a female it will make itself apparent in the character of a son or heterozygous daughter. In case it occurs in a male a heterozygous daughter will exhibit the new character. It is therefore not possible for a mutation to be transmitted unrecognized from one generation to the next.

3. *Procedure in those stocks in which facet counts were not made.* These stocks were kept with uniform food conditions at about 21°C. The bottles of each stock were arranged in a row in chronological order with a week's difference in time between two

adjacent ones. Each week the flies of the oldest bottle were transferred to a bottle with new food and the old bottle was discarded. The interval between the time that the flies were put into a bottle and the time of removal was three weeks. The length of the life-cycle at the chosen temperature is about fourteen days with a minimum of twelve, so that there was no possibility of the appearance of a second generation in any bottle.

In the examination of the stocks for mutants the flies were etherized and examined without counting the facets. The number of adult individuals was recorded. If no mutant was discovered, the flies were put into a bottle with new food according to the system given above. If, however, a mutant was discovered all the flies were discarded and a new bottle was prepared from the second bottle of the stock in case that did not have a mutant. A selection was thus made against stocks exhibiting mutation. There was no object in doing this other than the desire to preclude the possibility of recording a single mutant more than once.

The only chance of carrying a mutation forward would have come from a failure to recognize a mutant. In mutations from full to bar or ultra-bar the differences in both males and heterozygous females are too obvious to escape notice. In the reverse mutations from bar to full the males offer no difficulties, but in the unselected bar stocks heterozygous females coming from a low bar individual might rarely be confused with high bar homozygous ones.

In the case of ultra-bar to bar the degree of dominance of ultra-bar is so high that the heterozygotes are not readily distinguished from ultra-bar by simple inspection without facet counts and breeding tests. Here mutations might not be recognized until the second generation, when they would be readily observed because of the appearance of bar males. Male mutants are, however, readily recognized at once.

In the mutations from bar to ultra-bar there is usually no difficulty in recognizing the heterozygotes because the latter are closer to ultra-bar than to bar. Occasionally there may be a doubtful case in unselected bar stocks where the presence of

accessory factors for high facet number might make a heterozygote between bar and ultra-bar resemble a bar female with accessory factors for low facet number. As in the previous case, male mutants can be recognized at once.

TABLE 1

Full stocks examined for mutations to bar or ultra-bar. No such mutations were found. Temperature, 20° to 22°C.

NAMES OF STOCKS	CATALOG NUMBER	PERIOD OF OBSERVATION		NUMBER EXAMINED
		From	To	
Buff lozenge.....		1919 XI 6	1920 IV 19	1,295
Tinged furrowed.....		1919 XI 6	1920 IV 19	1,298
Wild.....	1119.1	1919 XI 6	1920 IX 15	3,535
Yellow body.....		1919 XI 6	1920 IV 19	555
Vestigial sepia.....		1919 XI 6	1920 IX 28	2,435
BPCPS.....		1919 XI 6	1920 IX 15	2,851
Forked.....		1919 XI 6	1920 IX 15	2,997
Full from 127 white unselected bar.....	1287.5	1920 I 3	1920 IX 13	4,117
Full from ultra-bar 499.42.....	1291.2	1920 I 27	1920 IX 10	3,577
Full from low F36 white bar	1212.1	1920 I 27	1920 VI 8	2,535
Full from low A red bar ¹	1309.5	1920 III 4	1920 X 1	3,312
Full from low F7 white bar	1308.3	1920 III 13	1920 IX 13	1,917
Full from low F41 white bar	1291.6	1920 III 15	1920 IX 10	2,559
Full from red bar 31.....	1314.3	1920 III 18	1920 IX 11	1,985
Full from BPCPS bar.....	1322.2	1920 III 18	1920 IX 13	2,817
Full from white emarginate.....	1308.2	1920 III 23	1920 IX 11	2,731
Full from high F28 white bar ...	1528.1	1920 IV 1	1920 IX 13	1,382
Full from high F5 white bar	1548.5	1920 V 22	1920 IX 14	1,505
Full from high F41 white bar ...	1549.7	1920 V 22	1920 VI 26	204
Full from low F38 white bar	1528.1	1920 V 22	1920 IX 14	933
Full from vestigial sepia bar....	1827.4	1920 V 31	1920 IX 15	1,550
Full from high A red bar.....	1870.6	1920 IX 7	1920 IX 15	100
Full from Morgan's broad bar ..	1861.1	1920 IX 10	1920 IX 15	100
Total.....				46,290

¹ A new mutant, 'oval full,' appeared in this stock.

II. DATA

1. Full-eye

Full-eye stocks of various kinds have been kept for about six years, but without counts of numbers of individuals observed except during the nine or ten months covered by table 1, when

more than 46,000 flies were examined. Throughout the whole period there was not a single case of mutation to bar or ultra-bar. Attention is called to the fact that various different kinds of stocks were used and that in the cases listed in table 1 the majority of the stocks are fulls derived by mutation from bar or ultra-bar. Their recent origin from the bar stocks might have led one to believe that they would possess a high degree of instability. A single 'oval full' mutation different from any of the bar mutations was the only change observed. The oval condition, however, is due to an accessory factor and is not in the bar allelomorphic series.

Full therefore shows no tendency to form bar or ultra-bar since the time of the single appearance of the former, as noted by Tice in 1913.

2. Bar eye

Tables 2, 3, 5, and 6 and figures 2, 3, and 5 give the data for bar eye. Attention is called to the great variety of bar stocks used, including unselected bars, high selected bars, low selected bars, reverse selections from high bar, bar mutations from ultra-bar, emarginate and a high or 'broad' bar recently received from Prof. T. H. Morgan. The differences between these various stocks are of two kinds: 1) differences in origin, some being derived by mutation from ultra-bar, while the majority came directly from full, and, 2) differences due to accessory factors.

The total number of observed individuals is 85,008. Among them there were noted 52 separate mutations to full, or one in 1636, which gives a mutation coefficient of 0.00061. There were also three mutations to ultra-bar, or one in 28,363, giving a mutation coefficient of 0.00003. It is probable that very few mutations to full were missed because of the ease with which full or heterozygous individuals may be distinguished from bar. Mutants from bar to ultra-bar may, however, be missed, as noted above, unless facet counts and breeding tests are made. The true number of these latter mutations may therefore be appreciably larger than is indicated in the table which includes only the tested cases. Among the mutations to full also a con-

TABLE 2
Mutations of bar stocks

NAMES OF STOCKS	CATALOG NUMBER	PERIOD OF OBSERVATION		NUMBER EXAMINED	MUTATIONS					
					To full			To ultra-bar		
					Females	Males	Total	Coefficient	Number	Coefficient
Unselected white bar. Parents of selec- tion lines ¹	127	1917 IX	23	1917 X	1	0	1			
BPCPS bar ²	1132.4	1919 XI	8	1920 X	1	0	1			
Red bar ³	23	1919 XI	8	1920 X	1	0	1			
Red bar ⁴	31	1919 XI	8	1920 X	1	2	3			
White bar ⁵	127	1919 XI	8	1920 X	2	1	3			
Unselected bar, total.....					6	3	9	0.00071	0	0.00000
High F ₄₁ white bar.....	473.1	1919 X	24	1920 X	1					
A red bar.....	336.1	1919 XI	15	1920 X	8	2	3			
B red bar ⁶	65.5	1919 XI	15	1920 IX	30	1	1		1	
F ₆ white bar.....	1561	1920 I	19	1920 IX	30	1	2			
F ₂₈ white bar.....	419.1	1920 I	28	1920 IX	30	0	3			
from special red bar 80 forked 1165.1.....	1543.7	1920 IV	17	1920 X	4	0	0			
White bar during the course of selection F ₁ to F ₄₂ ⁷		1917 X	5	1919 VII	15	4	0		1	
High bar, total.....					8	6	14	0.00063	2	0.00009

Low vestigial scopia bar.....	1147.1	1919 XI 8	1920 X 8	3,314	2	1	3			
A red bar ⁸	332.1	1919 XI 15	1920 VI 23	1,089	1	0	2			
F ₁₁ white bar ⁹	475.1	1920 I 21	1920 X 1	3,096	1	0	1			
F ₁₃ white bar.....	463.1	1920 I 29	1920 IX 18	3,160	0	0	0			
F ₇ white bar.....	168.1	1920 I 30	1920 IX 30	3,519	5	0	5			
from special red bar 80 forked										
1165.1.....	1961.5	1920 VII 20	1920 X 5	670	1	0	1			
White bar during the course of										
selection F ₁ to F ₄₂ 10.....		1917 X 5	1919 VI 20	6,662	2	1	3		1	
Low bar, total.....				21,510	12	2	15	0.00069	1	0.00005
Reverse selection from high F ₄₀ white bar.	469.3	1920 I 28	1920 IX 10	1,402	0	0	0			
F ₃₅ white bar.....	474.1	1920 I 28	1920 X 1	2,211	1	0	1			
F ₃₅ white bar.....	466.4	1920 I 28	1920 X 1	1,241	0	0	0			
Reverse selection from high, total.....				4,854	1	0	1	0.00021	0	0.00000
Bar from ultra-bar 1160.2 ¹¹	1303.1	1920 I 28	1920 X 5	2,821	1	1	2			
Special red bar 80 forked from ultra-										
bar ¹²	1165.1	1920 I 28	1920 IX 30	3,430	2	3	5			
Bar from ultra-bar 499.2.....	1291.4	1920 III 15	1920 X 4	2,386	0	0	0			
Bar from special red ultra-bar 45.....	1302.4	1920 III 15	1920 X 4	4,472	1	2	3			
Bar mutations from ultra-bar, total....				13,109	4	6	10	0.00076	0	0.00000

TABLE 2—Continued

NAMES OF STOCKS	CATALOG NUMBER	PERIOD OF OBSERVATION		NUMBER EXAMINED	MUTATIONS					
					To full			To ultra-bar		
					Females	Males	Total	Coefficient	Number	Coefficient
Red enarginate ¹³	1153.2	1919 XI 15	1920 X 8	5,924	0	1	1			
Bar from white enarginate ¹⁴	1308.2	1920 III 15	1920 X 1	3,620	0	0	0			
Broad bar ¹⁵	1861.1	1920 V 21	1920 IX 10	1,175	0	2	2			
Enarginate and other very high bars, total.....				10,719	0	3	3	0.00028	0	0.00000
Grand total, all bar stocks ¹⁶				85,008	31	20	52	0.00061	3	0.00003

¹ Temperatures were 20° to 22°C., except in the two selection lines which were 27°C. A 21-facet male and a 26-facet female may be ultra-bar and a 317-facet male may be enarginate, but all three died without fertile matings.

² A mutation to full which took place before the beginning of these observations is not counted.

³ A 400-facet male may be enarginate.

⁴ A 350-facet male may be enarginate.

⁵ A mutation to full preceding the beginning of these observations is not counted.

⁶ Accessory factors changing bar to enarginate have appeared several times in this stock.

⁷ Temperature, 27°C. A 13-facet male, a 19-facet female, and a few others may be ultra-bar, but no fertile matings resulted.

⁸ In one case there is uncertainty as to the sex of the mutant.

⁹ A mutation to full preceding the beginning of the observations is not counted. One low individual may be ultra-bar but it died before a test could be made.

¹⁰ Temperature, 27°C. 'Scab' eye appears frequently. A 21-facet male and a 36-facet female may be ultra-bar and some high males and females may be emarginate, but no tests were completed.

¹¹ Bar had completely replaced ultra-bar before the beginning of the observations.

¹² Distinct low and high lines were established from this stock.

¹³ There is one probable reverse mutation of the accessory factor producing emarginate.

¹⁴ Emarginate (mostly males) appeared at intervals, but the stock may not have been wholly free of emarginate factors at the beginning of the observations.

¹⁵ This stock was obtained from Prof. T. H. Morgan and consists of highs picked out from his bar stocks.

¹⁶ The sex of one mutant was not recorded.

siderable number were tested and were shown not to differ from wild full. Several of them serve as the starting-points of the full stocks listed in table 1 as derived from bar.

One of the most striking results is the uniformity in rate of mutation among the different kinds of bar stocks. There is no essential difference in this respect between unselected bar, high bar or low bar, or bar derived from ultra-bar (table 3). The only exception is emarginate eye (fig. 1, *E*, and table 2). The two emarginate stocks give only one mutant in 9544 individuals, giving a coefficient of 0.00010, though the number of observations is too small to give this value any special significance. Emarginate is a very high bar resembling in character a full

TABLE 3

Effect of origin and of selection for high and low facet number upon the rate of mutation in bar stocks

ORIGIN OF STOCKS	NUMBER OF INDIVIDUALS	TO FULL		TO ULTRA-BAR	
		Number of mutations	Coefficient of mutability	Number of mutations	Coefficient
Unselected.....	11,760	8	0.00068	0	0.00000
High selection.....	22,125	14	0.00063	2	0.00009
Low selection.....	21,510	15	0.00069	1	0.00005
Bar from ultra-bar.....	13,109	10	0.00076	0	0.00000

with a notch in one side. It has been shown to be due to accessory factors which are not sex-linked. A female heterozygous for the bar factor but retaining both emarginate factors is full-eye in appearance so that there is no possibility of mistaking a mutation of a bar factor to full. There does not seem to be any valid reason for expecting the presence of the emarginate factors in the autosomes or other factors present in the emarginate stock to influence the rate of mutation in the bar gene which is located in the sex chromosome. Until a larger number of individuals has been observed the low apparent rate of mutation in this stock may be ascribed to random sampling.

In the discussion of the mutations from bar special attention is called to the behavior of individuals undergoing selection for high or for low facet number, because it has frequently been stated

that the direction of selection has an influence upon the direction of mutation. In these experiments facet counts were made of all individuals so that the data have a high degree of accuracy. The results are shown in figure 2, in which the vertical scale is a logarithmic one so far as facet counts are concerned. A unit of the scale represents a factorial change which causes a change of 10 per cent in facet value or approximately the same as that produced by a difference of one degree Centigrade. The middle horizontal line is the mean value of the unselected population which serves as the zero of the scale. The upper horizontal line is the position of full eye on this scale and the lower horizontal line the position of ultra-bar. The upper zigzag line represents the fluctuation of the high selection line for forty-two generations and the lower zigzag line the fluctuation of the low selection line. The vertical dotted lines give the origins and directions of the observed mutations. It is to be noted that there is one mutation to full in the parental generation, *P*, before the beginning of selection and that there are four mutations to full and one to ultra-bar from the high selection line and three to full and one to ultra-bar from the low selection line. Since there are 14,327 individuals represented in the diagram with eight mutations to full, it follows that the mutation rate to full eye is 0.00056, which is of the same order as that of the whole group of bars, 0.00061. The four fulls in the high line and three in the low line are as close an approach to equality as can be obtained in this number.

The two mutations to ultra-bar are too few in number to furnish any accurate rate of mutation. The fact that two of the three observed mutations of this character come in the 14,000 individuals in which facet counts were made as opposed to one in the 71,000 individuals in which facet counts were not made makes it seem still more probable that, as indicated above, some mutants of this kind were overlooked in the later groups. It is interesting to note that one of the two mutations to ultra-bar in the selection lines came from the highs and the other from the lows.

The data at hand, in so far as they go, give no support to the view that direction of selection has an influence upon the direction of mutations.

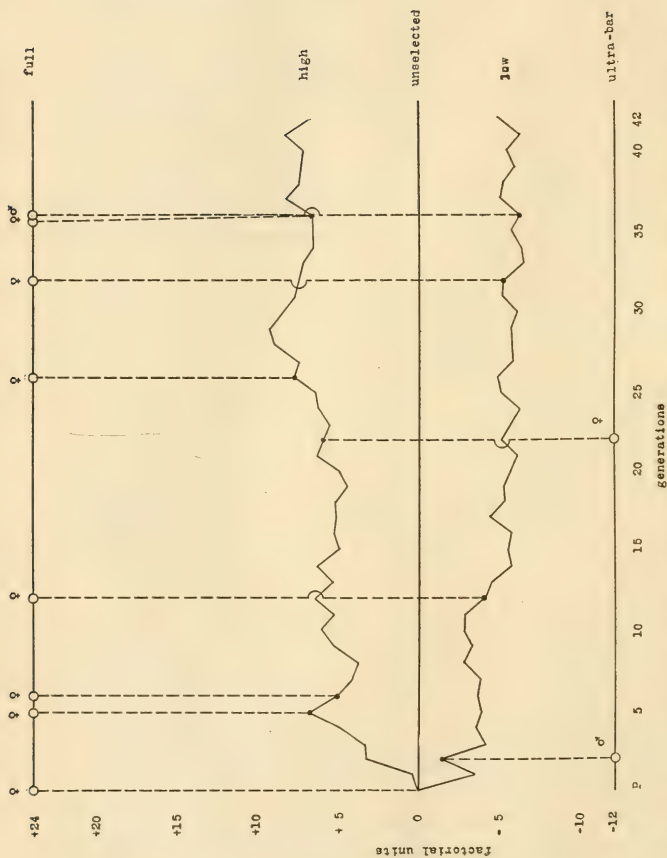


Fig. 2 Mutations to full and ultra-bar from white bar during the course of forty-two generations of high and low selection for facet number. The zero of the vertical scale is the mean of the unselected population and departures from it are logarithmic functions of facet number, a unit being equal to a change of 10 per cent in facet number. The upper zigzag line represents the course of the high selection and the lower zigzag line the course of the low selection. Mutations to full and to ultra-bar are represented by the vertical dotted lines.

3. *Ultra-bar eye*

The mutations from ultra-bar are given in table 4. The first two items, consisting of 3184 individuals, or over one-third of the whole, are made up of flies in which eye facet counts were recorded and in these sets the chance of overlooking mutations to bar is less than in the others. Nevertheless, there is no essential difference in the rates of mutation in the two groups, though the numbers are too small for any valid comparison.

Among the 8681 ultra-bar individuals examined there were five mutations to full, or one in 1726, which gives a coefficient of 0.00058. There were also three mutations to bar, or one in 2894, a coefficient of 0.00035. The reverse mutations to full are nearly as frequent as those of bar to full which have a coefficient of 0.00061. Reverse mutations to bar are more frequent than direct mutations from bar to ultra-bar which have a coefficient of only 0.00003. Notwithstanding the small number of mutations involved, the difference, though not its degree, may be a significant one.

In view of its origin from bar, it is a matter of special interest to note that ultra-bar may revert either to bar or directly to full.

The comparative frequencies of the various changes between full, bar, and ultra-bar are given in figure 3 and table 5. In figure 3 the observed jumps are indicated by arrows which are marked with the respective frequencies. The much greater frequency of the reverse mutations is apparent.

III. DISCUSSION AND CONCLUSIONS

1. *Are there definite and recurring stopping-places in mutation?* An examination of the data shows clearly that the germinal changes have definite and recurring stopping-places (fig. 3). In the present observations there are no demonstrated cases of intermediate conditions between the three components of the series. A possible exception was described by the author (Zeleny, '20, p. 308) as mutant D and has a dominance intermediate between that of bar and ultra-bar. It may be a case in which ultra-bar had mutated in the direction of bar, but not to the same point as

TABLE 4

Mutations of ultra-bar stocks

NAMES OF STOCKS	CATALOG NUMBER	PERIOD OF OBSERVATION		NUMBER EXAM- INED	MUTATIONS				
					To full		To bar		
		From	To		♀ ♀	♂ ♂	Coefficient	♂ ♂	Coefficient
Ultra-bar females. 27°C. ¹	800c	1917 XII 14	1918 VII 23	1,590	1	0	1	0	
Ultra-bar males. 27°C. ¹	800d	1917 XII 14	1918 VII 23	1,594	0	1	1	1	
White ultra-bar. 18°-24°C	499.42	1919 XI 21	1920 X 1	2,645	0	2	2	1	
Special red ultra-bar number 45. 18° to 24° C..	1214.1	1920 I 27	1920 X 1	2,852	0	1	1	1	
Totals.....				8,681	1	4	5	3	0.00035

¹ Several females intermediate between bar and ultra-bar (heterozygotes) were present, indicating an unobserved mutation in the male parent, and the entry is accordingly put in the male column.

TABLE 5

Frequency of mutation within the bar series of multiple allelomorphs

	NUMBER OF INDIV- IDUALS	TO FULL		TO BAR		TO ULTRA-BAR	
		Number of mu- tations	Coefficient	Number of mu- tations	Coefficient	Number of mu- tations	Coefficient
Full.....	46,290+	—	—	0	0.00000+	0	0.00000
Bar.....	85,008	52	0.00061	—	—	3	0.00003
Ultra-bar	8,681	5	0.00058	3	0.00035	—	—

the other bars. While it is probable that such intermediate members of the series may be definitely demonstrated in the future, it is clear that the recorded changes are between a few definite points and not from one point to any other point whatsoever. No such conclusion could have been reached if individual mutations had appeared only once or twice. In these observations the change from bar to full came fifty-two times and that from ultra-bar to bar five times.

A comparison with the white-eye series is interesting. Figure 4 gives all the changes observed in that series, according to Müller ('20). The difference between any two steps is so slight that there may be a question as to whether the components of

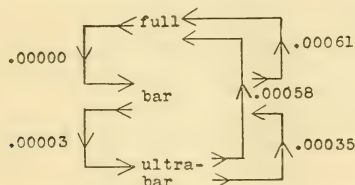


Fig. 3 The bar-eye series of multiple allelomorphs with the direction and frequency of all mutations. Note that the reverse mutations are much more numerous than the direct and that all three possible kinds of jumps in this direction are realized.

the series as given may not represent the limits of possible discrimination. According to the students of the series, however, there is never a doubt as to the classification of particular individuals, and this would seem to eliminate the view of existence of unperceived intermediates. The conclusion, however, is vitiated by the fact that most of the mutations have been observed only once.

2. What kinds of jumps occur between the three stopping-places?

An examination of figure 3 shows that five of the six possible kinds of jumps have occurred. The only one that has not been observed is that from full to ultra-bar. When it is considered that the jump from full to bar has not occurred at all in my material, the only recorded case being the original mutation as

described by Tice in 1914, and that the change from bar to ultra-bar has come only three times in 85,000 individuals, it is probable that the absence of the jump from full to ultra-bar is merely a matter of random sampling.

3. *The frequency of reverse mutations.* The special feature of the bar series is the frequency of reverse mutations (fig. 3). Each of the three possible reverse jumps occurs with a frequency much greater than that of the original mutations. The coeffi-

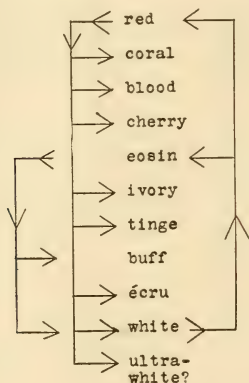


Fig. 4 The white-eye series of multiple allelomorphs arranged in order of darkness of color according to Muller ('20). All the observed kinds of jumps are indicated.

cients of the reverse mutations are 0.00035, 0.00061 and 0.00058 as opposed to 0.00003, 0.00000 +, and no recorded mutations. Since the original observation of reverse mutations by May they have been noted in the white eye series of *Drosophila* where several returns of white to eosin and one of white to red have been noted. In none of these other cases do the reverse mutations occur with the great frequency noted in the bar series.

It is obvious that the presence and absence theory cannot apply to these changes. The closest analogy seems to be to chemical changes involving a rearrangement of atoms. In the bar series the rearrangement in one direction is more easily accomplished

than in the other direction. The relative frequency of reverse mutations as compared with direct ones shows that the stability of the material composing the gene is much greater at the full-eye position than at any other. At the bar-eye position, where mutation occurs in both directions, the return to full occurred fifty-two times as opposed to three changes in the other direction to ultra-bar, though the latter number probably is incomplete because some heterozygotes may have been overlooked in that part of the observations in which facet counts were not made.

4. *Is recent origin an explanation of the frequency of reverse mutations?* It might be supposed that the frequency of mutations in the direction ultra-bar to bar to full is due to the fact that bar and ultra-bar have originated recently and are therefore relatively unstable. If this were a true explanation it would apply equally well to full-eyed stocks recently derived by reverse mutation from bar or ultra-bar. As shown in table 1, there is not a single case of return to bar or ultra-bar in such stocks. The same consideration applies to bar derived from ultra-bar. It does not furnish a single case of return to ultra-bar (tables 2 and 3).

5. *Are shorter jumps more frequent than longer ones?* In the direct mutations the original ultra-bar came by two steps from full. The number of observed mutants, however, is too small for any conclusion as to relative frequency of short as compared with long jumps.

Among the reverse mutations there are five from ultra-bar to full in 8681 individuals, or a coefficient of 0.00058. Bar to full appears in fifty-two cases among 85008, or a coefficient of 0.00061, and ultra-bar to bar in three out of 8681, or a coefficient of 0.00035. It is clear that these data furnish no support for the view that shorter jumps are more frequent than the longer ones. Until more adequate data are obtained the conclusion may be drawn that there is no essential difference between the two.

6. *Does direction of selection have an effect upon direction or frequency of mutation?* It has been claimed that the direction of selection has an influence upon the direction of mutation. According to this view, there should be a preponderance of

upwardly directed mutations in a high selection line while in a low selection line the downwardly directed ones should be in the majority. This view is supported by May's data, in which eight of the nine mutations to full appearing in the bar-selection experiments were in the high lines. The exceptional one appeared during reverse selection from a low line.

The present data obtained in the course of forty-two generations of selection from high facet number on the one hand and low facet number of the other are given in figure 2. There is clearly no significant difference between the high and low lines in the frequency of mutation either to full eye or to ultra-bar eye. There are four mutations to full in the high line and three in the low line, and ultra-bar appears once in each line. A final conclusion on this point must of course await more extensive data, but attention is called to the fact that the provisional conclusion drawn here is supported by the findings regarding rate of mutation in stocks derived from the selection lines as given in the following paragraph.

7. *Does high or low facet number in a stock have an effect upon the direction or frequency of mutation?* High stocks were obtained from the high selection lines and low stocks from the low selection lines. Such stocks have remained high and low, respectively, when bred without further selection. One of the very high stocks, emarginate, arose by mutation during upward selection.

It has been supposed by a number of students of evolution that a high stock has a greater chance of reaching any particular higher point than does a low stock, because the distance to be traveled is less. As stated above, it has been demonstrated for the bar series that within the bar locus there is no greater frequency of the short than of the long jumps. The germinal differences to be considered under the present heading are, however, not at the bar locus. As stated elsewhere (Zeleny, '18) and demonstrated more fully in a paper on the effects of selection which is to be published shortly, the high bar stocks discussed here differ from the low ones in their accessory factors. As shown in table 3, there is no significant difference either in direction or infrequency of mutation between high and low bar stocks. The

coefficients of mutation in unselected, high and low stocks are, respectively, 0.00068, 0.00063, and 0.00069. There is thus no evidence that the presence or absence of particular accessory factors affects the frequency of mutation.

The emarginate stock (fig. 1, *E*) is a very high stock derived from bar by mutation in an accessory gene. As shown in table 2, it has an unusually low mutation rate to full. If an effect of an accessory factor upon mutation at the bar locus is assumed in this case, it is in the opposite direction from that usually postulated.

8. *Does direction of origin have an effect upon the direction or frequency of mutation?* There is a widely prevalent view that biological states cannot be described in terms of present condition alone, but that a knowledge of past history is necessary for the prediction of their reactions. They are supposed to retain the ear-marks of their past. Perhaps such views merely indicate a general appreciation of the complexity of biological phenomena and the feeling that for that reason there cannot be identity of present structure and behavior when past histories are different. This is especially true in the discussions of the probable course of evolution and is one of the postulates of orthogenetic theories. On the other hand, chemists and physicists are inclined to consider a particular substance as being always the same, regardless of its source, though an outsider may notice a present tendency to scrutinize carefully supposedly identical substances derived from different sources.

The different components of the bar series are definite entities comparable to definite chemical compounds or physical states. This is indicated by the facts of their structure, their behavior in heredity, and their tendency to mutate in the same manner regardless of the character of their origin. Thus bar derived from full is not different from bar derived from ultra-bar, and full derived by reverse mutation from bar or ultra-bar is not different from the original full.

9. *Is there a periodicity in the mutations?* It is common belief that mutations are periodic, occurring with great frequency during a limited time and very rarely or not at all at other times. The studies of *Drosophila* as a whole have not yet demonstrated

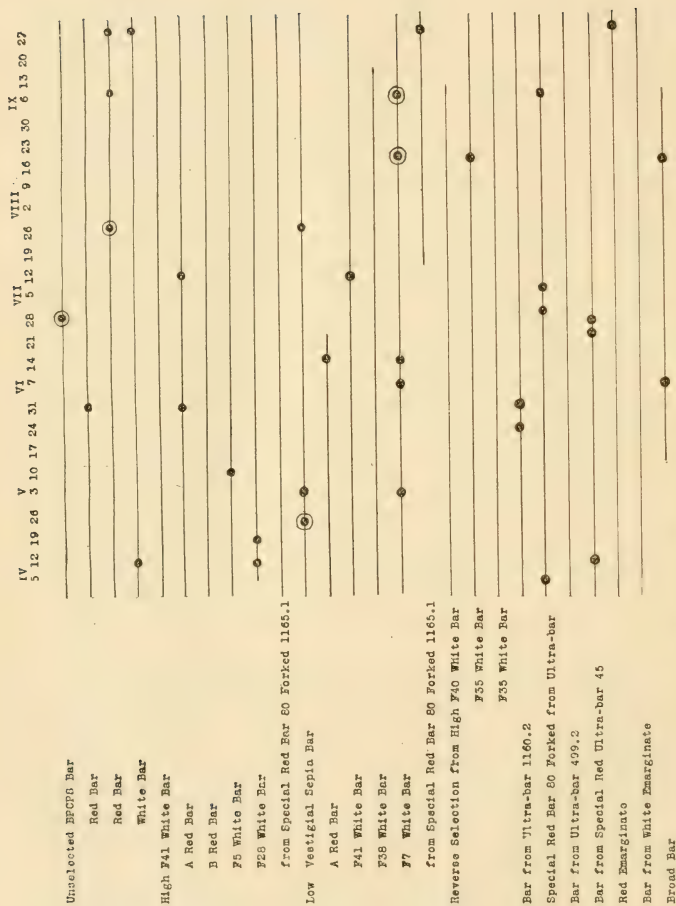


Fig. 5 Chronological arrangement of mutations from bar to full for each of the stocks under continuous observation from April 5 to September 27, 1920. ●, single mutant individual; ⊙, more than one mutant individual.

any such periodicity. However, there have been no adequate data bearing on a possible periodicity in appearance of a particular single mutation. This has been due in part to the infrequency of most mutations and in part to lack of concentrated attention upon single mutants for considerable periods of time.

In the present series of observations the number of mutations observed at a single locus is considerable, but the period of time is comparatively short. In the observations made upon the stocks shown in figure 5 large numbers of individuals were observed during a period of six months. The mutations are distributed fairly uniformly throughout this period. In the case of the selection lines shown in figure 2 the observations extend over twenty-two months, but include a much smaller number of individuals than the other series. An examination of both series of data and a consideration of the fact that from the time of its origin bar has been subject to 'contamination' by full leads to the conclusion that no periodicity has been demonstrated.

In this connection it is well to consider that the environmental conditions were kept as uniform as possible, so that there is no basis for a periodicity due to environmental periodicity. The bearing of the results is upon the possibility that the germplasm undergoes periodic changes in condition independently of the environment. No such changes have been demonstrated. This uniformity in the germplasm is in line with the results of comparisons between rate of mutation in stocks of different origin and in those with different accessory factors, neither of which seem to affect the direction or rate of mutation.

10. *Does mutation occur in both male and female parents?* If the internal change in a sex-cell producing a mutant in this series occurs in the male parent, a heterozygous daughter results. If it occurs in the female parent, either a mutant son or a heterozygous daughter appears. If the change may occur in either parent, both heterozygous daughters and mutant sons are to be expected, but with a preponderance of the heterozygous daughters over the full sons. If it is postulated that an X chromosome has the same liability to mutate whether located in a male or in a female, the chance of mutation in any individual female sex-cell

is twice as great as in the male, since oogonia have two X chromosomes and the spermatogonia only one. This is true not only in case the mutation of single chromosomes is independent of their location, but also in case mutations are due to environmental forces acting equally in male and female. In the latter case there would be simultaneous mutation of both chromosomes in the female. For the sake of simplicity in exposition, it may be assumed that the mutations occur during the growth period preceding maturation, though the result would be the same if the change occurred at any time prior to this. If one of the two sex chromosomes of the primary oocyte contains a changed gene, the chance is one in two that the mature ovum will obtain it. If the single sex chromosome of the primary spermatocyte contains a changed gene, one-half of the four resulting spermatozoa will contain it. It follows on the hypothesis of equality in rate of mutability of individual bar genes that there should be twice as large a proportion of eggs with a changed bar factor as of changed spermatozoa. This result, as stated above, is independent of the period during the gonial divisions during which the mutations appear.

Of the eleven mutants from bar to full observed by May ('17) six were full males and five heterozygous females. Assuming the whole number of males and females examined to be equal, this approach to equality in the number of mutants seemed to favor the view that the mutations are confined to the females. This conclusion led May further to consider the possibility that the changes from full to bar and of bar to full may be cases of partial nondisjunction. This latter hypothesis is improbable on other grounds which need not be discussed here.

Taking all the valid cases of mutation from bar to full in the present report, there are thirty-one heterozygous daughters and twenty full sons among the mutants. In these observations there was no chance of overlooking full sons, but heterozygous daughters derived from low individuals in unselected stocks may have been overlooked because of their closeness to high-bar homozygotes. Heterozygous females may likewise have been overlooked in May's observations. This larger number of cases therefore supports the view that the mutations may occur in

either male or female sex-cells. The conclusion is based on the assumption that approximately equal numbers of males and females were observed. In most of the present counts males and females were not listed separately, but other counts indicate that while there may be a wide departure from equality in individual bottles the departures occur in both directions, and in a large series of observations the number of males and females approaches equality. In one part of the present observations, the selection lines, accurate records were kept of the sex of all individuals. Here there are 7189 females and 7138 males, a close approach to equality. Of the eight mutants to full in these selection lines seven are females and only one a male. This ratio distinctly favors the view that mutations may arise in male sex-cells as well as in the female.

The other mutations in the bar series do not throw any light on the present question because of the probability of missing heterozygous females. In fact, they give fewer females in proportion than is to be expected on the hypothesis that the mutations occur in the female sex-cells alone. The only recorded mutation from full to bar appeared in a son. Of the five mutations from ultra-bar to full only one is a daughter, while four are sons. Of the three mutations from ultra-bar to bar all are sons. Finally, of the three mutations from bar to ultra-bar one is a daughter and two are sons.

It is interesting to speculate further on the degree of independence of the changes in the bar gene. For instance, is the bar gene of one chromosome in the female independent in this respect of the bar gene in the other or do they change simultaneously? Unfortunately, no satisfactory test of this proposition has been devised for the bar series. Muller ('20) has, however, pointed out that mosaic mutants involving recessive sex-linked genes are always males and that this indicates that mutations occur in only one member of a pair of chromosomes at a time.

There is another question of equal interest which may perhaps be capable of solution. Does the change occur with equal readiness in all individual animals? This cannot be tested in females,

because the number of fertile eggs is too small to give an expectation of mutation in every individual. Among males, however, the number of spermatozoa is sufficiently large to give an expectation of several mutations in each individual male in case a considerable percentage of the spermatozoa are made effective in fertilization. It may be possible to test such a hypothesis by mating a single male to a large number of females.

11. *At what time in the life-cycle do the germinal changes appear?* In case a mutation occurs after the last gonial division, only a single affected ovum or two affected spermatozoa are to be expected. If it occurs before the last gonial division, the number of affected eggs or spermatozoa is greater the earlier the appearance of the change. Therefore, it may be argued that the number of mutant individuals appearing at one time is a rough indicator of the period at which the mutation occurred. This procedure is obviously subject to two sources of error. In the first place, it is possible that there may be two independent mutations appearing in the same generation. Since the number of individuals appearing in a single bottle rarely exceeded 300 while the rate of appearance of mutations is once in 1500, the chance that two independent mutations will appear in the same bottle is not more than one in five squared or one in twenty-five. The second source of error is more serious. A single adult mutant may be merely the remnant of several mutant sex-cells. What appears to be a mutation appearing late in gonial history may in reality have come much earlier.

In general it may be argued that the simultaneous appearance of two or more individuals indicates with a fair degree of certainty that the germinal change occurred early in oogenesis or spermatogenesis. The appearance of a single mutant individual, on the other hand, does not establish the occurrence of the change as coming late in gonial history. It merely makes it improbable that the change came near the beginning of that period.

Of the forty-four separate mutations which are suitable for the present purpose and which appeared in the stock bottles, thirty-nine came as single individuals and only five as more than one individual. In figure 3 the dots surrounded by circles represent

cases with more than a single individual. Table 6 gives the number of mutant females and males appearing in each of these five cases. In the selection lines, as shown in figure 2, each of the eight mutations appeared as a single individual. There is thus a great preponderance of single mutants.

The facts in the case make it necessary to conclude that at least five of the mutants represent changes occurring before the end of the gonial divisions, probably early in oogenesis or spermatogenesis. On the other hand, it is not possible to draw any definite conclusion regarding the relative frequency of the late appearance of germinal changes because a considerable number of the single mutants are probably merely chance survivors among a group of similar sex-cells. However, because of the

TABLE 6

Bar stocks in which more than one mutant individual to full appeared simultaneously

NAME OF STOCK	NUMBER OF HETERO- ZYGOUS FEMALES	NUMBER OF FULL FEMALES	NUMBER OF FULL MALES
B P C P S Bar 1132.4.....	Several	0	Several
Vestigial sepia bar 1147.1.....	3	0	3
Unselected bar 31.....	1	0	1
Low white selection F7 168.1.....	2	0	0
Low white selection F7 168.1.....	6	0	7

large number of such single mutants, it is probable that at least some of them represent mutations late in oogonial or spermatogonial history or during the growth or maturation periods. The conclusion may therefore be reached with a considerable degree of certainty that the mutations in the bar series may occur either early or late in gonial history.

It remains to consider the probability that both the affected spermatozoa resulting from a single mutation occurring during maturation may have given rise to mutant individuals. Such spermatozoa would necessarily have to give rise to heterozygous females, since they carry the sex chromosome and since the chance of meeting a mutant ovum is only one in 1500 squared. One of the five cases with more than a single mutant individual consists of two heterozygous females and may be due to the survival

of both the affected spermatozoa. In view of the small percentage of spermatozoa that survive, such an explanation, however, is improbable.

12. *Are mutations at the bar locus more or less frequent than those in accessory factors affecting eye-facet numbers?* Where more than one germinal difference affects the same somatic character it has been found in studies of other characters than bar that these differences are more frequently at various loci than at a single one. While the data on accessory factors affecting facet number are to be given elsewhere, it may be stated here that all the observed mutations in the accessory factors together are not as numerous as the changes at the bar locus. In this respect bar differs from the general rule as stated above. On the principle that there are several loci outside of bar and therefore several materials whose change affects facet number, it might have been supposed that there would be that much more opportunity for change in some one of them than in the single material at the bar locus. The material at the bar locus therefore has a higher degree of instability than the materials at the loci of the different accessory factors.

13. *Do the three genes at the bar locus form a quantitative series?* This question naturally arises in connection with a discussion of the origin of mutations. Ultra-bar may presumably be considered as due merely to the addition of another bar factor to the previous one. In a former paper (Zeleny, '20) it was pointed out that a comparison of the facet values of 1) full, 2) full x bar, 3) bar, 4) ultra-bar x bar, and 5) bar females should throw some light on the correctness of the quantitative theory. The five kinds of females should, according to this theory, differ by equal amounts of a single germinal material and there should be some constant relation between their facet values. No such regular gradation in facet values is found. Furthermore, ultra-bar males should have a value approximating that of bar females and bar males a value similar to that of heterozygous females. This also is not true. Therefore, it seems necessary to assume that the two steps in the series are qualitatively different or at least are not of the same order.

14. *Is the rate of mutation affected by the temperature?* Muller and Altenburg ('19), in a study of the frequency of appearance of lethal factors in the cap chromosome of *Drosophila*, raised some of their families at about 66°F. (18.9°C.) and others at about 80°F. (26.7°C.). The 445 families raised at the lower temperature produced five lethals, or one in ninety, and the 517 raised at the higher temperature gave thirteen lethals, or one in forty. This gives a positive value of two to three for Q_{10} .

The present data on mutation of bar to full furnish some evidence on the temperature effect. The two selection lines were kept at 27°C. All the other stocks were kept at 20° to 22°, except that the temperature dropped somewhat lower on a few occasions when the flies were kept in a cooled chamber during the summer. The difference between the temperature of the two sets is about 6°C. At the lower temperature there were forty-four mutations from bar to full in 70,681 individuals, or a mutation coefficient of 0.00062. At the higher temperature there were eight similar mutations in 14,327 individuals, or a mutation coefficient of 0.00056. The difference between the two cannot be considered as significant and is not even in the same direction as that obtained by Muller and Altenburg. The present data are subject to the criticism that the comparison is between stocks not undergoing selection on the one hand and those in process of selection on the other. The objection, however, is not a valid one because high and low selection lines during the process of selection, high and low stocks derived from these lines, and unselected stocks, all have approximately the same mutation coefficients. It has seemed desirable, however, to test the temperature relations to rate of mutation more fully and a carefully controlled series of experiments is under way.

IV. SUMMARY

1. The bar-eye allelomorphic series of *Drosophila melanogaster* is especially favorable for studies of the nature of mutation.

2. There are three definite stopping-places without intermediates.

3. All possible kinds of jumps between these stopping-places have been observed except full to ultra-bar.

4. Reverse mutations are more frequent than the original direct ones.

5. Recent origin is not an explanation of frequency of mutation, because full derived by mutation from bar or ultra-bar does not revert.

6. The longer jumps are as frequent as the shorter ones.

7. The direction of selection for facet number has no effect upon the direction or frequency of mutation.

8. The presence or absence of accessory factors causing high or low facet number in bar stocks does not affect the direction or frequency of mutation of bar.

9. The direction of origin of bar has no effect. Bar stocks derived by reverse mutation from ultra-bar show the same direction and rate of mutation as bar stocks derived directly from full.

10. There is no evidence of periodicity in the mutations.

11. Mutations occur in the germ plasms of both males and females.

12. Mutations are not confined to a single period in the germ-cell history. Some appear early and others late in oögenesis or spermatogenesis.

13. Frequency of observed mutations at the bar locus is greater than in all accessory factors combined.

14. The changes in the gene which produce the somatic series full to bar to ultra-bar are probably not of a quantitative nature.

15. The different components of the bar series are definite entities comparable to definite chemical compounds or physical states.

16. There is no difference in rate of mutation from bar to full between stocks kept at 20° to 22°C. and those kept at 27°C.

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Resumen por el autor, Vasil Obreshkove.

Las reacciones fóticas de los renacuajos en relación con la ley de Bunsen-Roscoe.

Los renacuajos de *Rana clamitans* que han permanecido durante algún tiempo en la oscuridad cuando se les ilumina súbitamente con una luz de suficiente intensidad responden al estímulo avanzando rápidamente hacia delante. El tiempo que transcurre entre la aplicación del estímulo y la respuesta varía con la intensidad de la luz. A causa de esta relación definida el autor supone la existencia de una substancia fotoquímica presente en los fotoreceptores, cuya substancia sufre ciertos cambios definidos durante la iluminación. El autor ha llevado á cabo un estudio para determinar hasta que punto la ley de Bunsen-Roscoe es aplicable á los cambios que tienen lugar en los fotoreceptores.

La razón de las intensidades extremas empleadas fué 1:500; comparándose la sensibilidad del ojo con la de la piel y también con la sensibilidad cuando se iluminan ambos órganos. Los cambios experimentados en los fotoreceptores durante la iluminación proceden de acuerdo con la ley de Bunsen-Roscoe. Con una serie media de iluminaciones, los experimentos demuestran que los productos de la intensidad por el tiempo coinciden. Con mayores intensidades tiene lugar una desviación en los productos de la intensidad por el tiempo, la cual es gradual y parece proceder con una constancia definida. El autor presenta una representación empírica, la cual satisface bien los datos actuales obtenidos. El proceso de la fatiga y el recobro de la sensibilidad fotosensorial son también objeto de discusión.

THE PHOTIC REACTIONS OF TADPOLES IN RELATION TO THE BUNSEN-ROSCOE LAW

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I. INTRODUCTION

The principle of transference of energy implies that for a given amount of energy used in the incitement of a reaction there must be evoked an equivalent effect. This principle has led to the development of a number of laws in general chemistry, which furnish expressions of the relation of the amount of energy received, to the effect produced, and the progression of this effect with the time of action and active masses.

In 1862 Bunsen and Roscoe showed that in order to produce equal shades of darkness on silver-chloride papers exposed to

various intensities of illumination, the time required was inversely proportional to the intensity of the light. The intensity of light and the time of action varied in such a manner in the production of the same effect that the product of these two variables was always constant. This can be expressed:

$$I \times T = K,$$

where I stands for the intensity of light, T the time of action, and K the constant effect produced.

Much has been written regarding the responses of animals to light, and it is now believed that light acts as a stimulus to the photoreceptors through a change in a chemical substance with which the nerve endings are in immediate contact. Parker and his students have described a number of fishes and amphibians possessing photoreceptors in the skin. This work has been recently extended to animals belonging to other groups. It seems advisable, therefore, to undertake a study with the aim of determining (1) to what extent the Bunsen-Roscoe law is applicable to animals which respond to light; (2) the dynamic nature of the responses, and, (3) the extent to which chemical changes produced in receptors are analogous to those in general chemistry.

In sensory reflexes, however, the apparent effect produced—let us say as a change in a specific peripheral receptor—is preceded by several other steps, namely: 1) a diffusion of the substance newly formed in the receptor to the specific nerve endings; 2) transmission of the impulse from the nerve endings to the adjustor and thus to the muscles, and, 3) contraction of the muscles. When in the test of the validity of the transference of energy, these intervening steps occupy a relatively long period, they must be separated from the energy-receiving period, analyzed, and appropriate values given to them.

We have reason to believe, however, that a species whose responses are characterized by rapid changes in the receptors after stimulation and a very short secondary period is an animal better adapted to receive and respond to a specific stimulus than one in which these changes are slow. When the specific

energy involved is small in amount, a critical quantitative study of the responses may enable us to substitute for the terms 'strongly phototropic' and 'slightly phototropic,' heretofore used in describing degrees of sensitivity, a numerical value for the intensity-time product. A series of such studies on various organisms, if successful, should enable us to arrange them in the order of their photosensitivities. This in turn may throw some light on the organization of the animal and on the extent to which this state serves it in its life.

Quantitative studies have already been made by Hecht ('18, '19a, b, '20) on the dynamic nature of the responses of *Ciona* and of *Mya*. The animals used in the present study were tadpoles of *Rana clamitans*. These were especially appropriate in such a study, since they remain undisturbed in the dark for long periods, and in sudden illumination produce clear-cut responses by forward swimming or by movements of the tail.

My thanks are due Prof. G. H. Parker, who suggested this problem and supervised the work and for whom, both as teacher and as adviser, I have the most sincere respect. His characteristic way of making suggestions and offering criticisms has served as an inspiration during the time of my association with him.

II. HISTORICAL REVIEW

Interest in the photodynamic behavior of animals and plants and the extent of agreement with the Bunsen-Roscoe law has been shown only comparatively recently. Blaauw ('09) was one of the first to undertake an investigation of this subject. In the production of a curvature in the seedlings of *Avena sativa* when exposed to light of various strengths, he found that the value of the product of the intensity of light and the time required to cause the same number of the plants to bend toward the illuminated side were nearly constant. Similar observations have been recorded by Fröschel ('09) with work on other plants.

Loeb and Northrop ('17) used the larvae of barnacles. These animals were found to be strongly phototropic. When lights

of equal intensity were placed one on each side of a rectangular aquarium containing the larvae of barnacles, collected in a blackened pipette, the animals when freed oriented themselves in their swimming in such a manner as to form a trail at an angle of 90° to a line connecting the two lights. When the lights were of unequal intensity, the trail formed was toward the weaker light. The degree of deflection was found to be dependent upon the ratio of the two intensities used, thus showing in a relative way that the effect produced is dependent on the amount of energy received.

Loeb and Wasteney ('17) experimented with *Eudendrium*, and the results were similar to those obtained by Blaauw.

In some recent publications Hecht ('18, '19a, b, '20) has recorded a series of readings representing the photic sensitivity of *Ciona intestinalis* and *Mya* when the time of exposure and the intensities of light were varied. The results obtained leave no doubt that the velocity of change in the receptors during illumination is dependent upon the intensity and the time of action. He has pointed out that to produce in an animal, a reflex due to light the time required is inversely proportional to the intensity of the light and therefore that the product of these two is a constant quantity for all conditions.

III. MATERIAL AND METHODS

Tadpoles of *Rana clamitans* are very abundant in the ponds in the vicinity of Cambridge and Boston. The tadpoles transform the next season after hatching, thus covering two breeding seasons. Hence during any month of the year there may be found in the ponds tadpoles of various sizes; some that were hatched during the preceding spring and others that are about to transform.

Animal dealers in Boston keep the tadpoles of *Rana clamitans* for sale throughout the year, and from these sources the greater part of the material for the experimental work was secured. On several occasions, however, the animals were brought directly from the ponds into the laboratory. Such tadpoles usually were found much overfed and inactive and

were not favorable material for immediate experimental work. These individuals were kept in the laboratory for at least one week before they were subjected to experiment. In that time the mud and other matter which filled the stomach and intestine when the animal was captured were discharged and the animals became accustomed to the laboratory condition, were easily stimulated, and reacted with regularity to light.

1. Selection of animals for experimentation

Although the melanophores in the tadpoles of *Rana clamitans* are not subject to as much contraction and expansion as is observed in other tadpoles and in certain fishes, yet during the course of the experimentation, as well as when tadpoles were just brought from the pond or from the general aquarium, they showed considerable variation in the state of these bodies. A knowledge of the condition which induces contraction and expansion of the melanophores and the general state of the animals when in either condition proved of importance early in the work since tadpoles darker in color from expanded pigment cells showed a slower reaction time than those that were lighter in color. It was decided, therefore, that that condition of the melanophores is an important factor to be kept in mind for a study like the one undertaken, where the reaction-time of the individuals to light constituted the only measurement for the examination of the validity of a law.

Tadpoles caught in the shallow water of a pond where the background and bottom were chiefly white clay, and tadpoles which had been left in white porcelain dishes in a light room for several days were found to be light in color. On the other hand, tadpoles which were captured from deep portions of the pond, and had been concealed under grass or debris, were dark brown in appearance. Light colored tadpoles, however, which had been placed in 0.2 per cent chloretone solution for anesthetizing, preparatory to the removal of the eyes, expanded the melanophores in the course of a few minutes. Such animals, when placed in the dark room, remained dark for several days, and in

most cases this condition was retained for much longer periods. Light-colored tadpoles, however, which had not been subjected to anesthetics, when removed to the dark room, remained in the light condition. The work was confined, therefore, to tadpoles as nearly as possible of the same color, and those somewhat pale in appearance were chosen. For this reason, when blind animals were needed for some phases of the problem it was found advisable to remove the eyes without previously placing the individuals in chloretone. It is very possible that the slower reaction-time of animals with expanded melanophores is due to a nervous condition of the individual which may be associated with the melanophores in an expanded state. There is another and more plausible explanation of this condition, which will be considered in connection with another phase of the problem.

Preliminary experiments revealed the necessity of avoiding another difficulty. Individuals differing in size showed variation in the regularity with which they responded. The smaller individuals were more active and more orderly in their responses. Tadpoles between 4 and 5 cm. in length were found to be the most favorable.

Tadpoles which were chosen for the experimental work on the basis of size and coloration were kept in the darkroom throughout the period of experimentation. Previous to each experiment, however, they were tested to determine whether they were in normal state, free from any infection, such as saprolegnia, and whether all of them were of equal photosensitivity. This was done in the following manner. Five or six tadpoles were placed in a white porcelain dish about 37 cm. in diameter and with just sufficient water to cover them. Such individuals were then repeatedly stimulated with a constant intensity of light at intervals of one minute or longer. Only those tadpoles were chosen which, with no exception, showed activity upon the application of the light. Those which showed indifference to the light stimulus were removed and placed in a special dish and from time to time tested during the next few days. These tadpoles usually died soon after being isolated from the chosen stock. Previous to each experiment the selected animals were

observed in the dark with the aid of a constant dim light, to determine the frequency of the spontaneous movements. When their state of activity was such as to leave no doubt that their movements were due to the applied stimulus of light, the experimental work was begun. This usually took place when the time of rest in the dark was several times longer than the time required to produce a reaction after the stimulus had been applied. Then any movement after the stimulus was applied could not be questioned as having been the result of the effect of light.

2. Apparatus

The apparatus for the first set of experiments (fig. 1) consisted of a small rectangular table (a, b), on which was placed a bicycle tire (c) and then a rectangular board (n). On this rested a round white porcelain dish (v) of the size already mentioned, containing a number of tadpoles of equal photosensitivity as previously determined. A short distance in front of the table and slightly above the level of the vessel was a dim light of 1 candlepower inclosed in a box (q). The front of the box was made of a number of layers of thin translucent paper, which allowed just sufficient light to escape for the observation of the animals. The legs of the table rested upon individual mats (m) each formed, from the floor up, of, 1) a rectangular rubber plate 3.5 cm. in thickness; 2) a tile of the same size and thickness; 3) a pad of excelsior, and, 4) a small square board. It was believed that such an arrangement would absorb any vibration which might occur during the experimentation. The light (s) was from above. By using a flash-light bulb of 0.5 candlepower and Mazda tungsten electric bulbs of 10 and 40 watts and by varying the distance of the bulb from the tadpole, a series of intensities was secured ranging from 0.3 candle-meter to 500 candle-meters. The amount of light which these bulbs produced was accurately measured by means of a Lummer-Brodhun photometer.

3. Method

If tadpoles subjected to various intensities of light are responsive with any degree of regularity, they ought to furnish measurements of the photic reactions for an examination of the Bunsen-

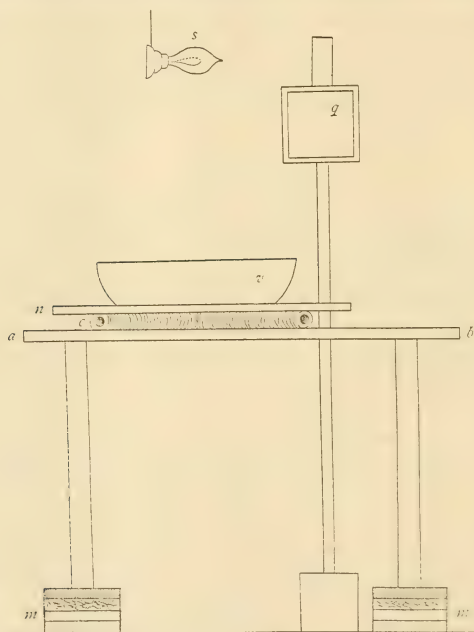


Fig. 1 Diagram of apparatus used for testing the sensitivity of tadpoles of *Rana clamitans* to various intensities of light. *a, b*, small rectangular table; *c*, bicycle tire; *n*, rectangular board; *v*, porcelain dish; *q*, dim light inclosed in a box; *m*, mat; *s*, source of light.

Roscoe law, especially if any relation between the degree of stimulation and the time which elapses before a response is noted can be ascertained. The intensities generally used were 0.3, 0.71, 1.2, 5, 10, 15, 20 and 30 candle-meters. To these, in the latter part of the work, were added 60, 200, and 500 candle-

meters. In the first set of experiments the photosensitivity of tadpoles with eye and skin was tested.

The use of such low degrees of stimulation seems to be in marked contrast with the methods used in the study of the reactions of amphibians to light by other workers. A review of the extensive literature on the subject reveals that the intensities used in various investigations range approximately from 200 candle-meters to 1000 candle-meters, and in some cases much higher degrees of stimulation were used. The instances where low intensities were used are few, but in all cases the object of the studies was to test the orientation of the animals in relation to a source of light and no attempt was made to determine extensively any quantitative relation which might exist between the degree of stimulation and response. Cole and Dean ('17), working with the tadpoles of *Rana clamitans*, observed photokinesis when three intensities were employed, 500 c.p., 100 c.p., and 48 c.p. (distance from source of stimulus was not given). The present study revealed that, in addition to this, these tadpoles, when kept in the dark and then suddenly and repeatedly illuminated by relatively low intensities within a certain range, show a period elapsing between the moment of stimulation and response which can be accurately measured by a stop-watch. This can be called the reaction-time and includes a sensitization period, during which light energy is taken up by the receptors and thus initiates a change which acts as stimulus to the nerve endings, and also the time for certain secondary processes, such as diffusion in the receptors to the nerve endings, transmission of the impulse to the adjustor and effector and the time occupied by the muscles in their contraction. This secondary period involving the processes just enumerated is discussed by certain authors under the term of 'latent period.' Others use this term to designate the total time from the application of the stimulus to the observable response. In this discussion we shall use reaction-time to designate the time occupied by sensitization, that is, the minimum period during which the light must act in order to produce a change in the receptors of sufficient strength to initiate a reflex, plus the time occupied by any secondary processes.

If animals that were to be repeatedly stimulated were allowed one minute of rest in the dark before stimulation was renewed, it was found possible to secure from fifteen to twenty readings from the same individual without any evidence of exhaustion. Now and then in the course of the experiment inconsistencies occurred. These were of the type of premature response or delayed response in the midst of a series of readings otherwise characterized by marked regularity. These irregular responses were recorded, however, and it will be evident from an inspection of the results that they were not numerous enough to throw doubt on the numerical values obtained. An insight into the cause of these occasional irregularities is difficult to obtain. Premature responses were associated at times with discharges from the intestine.

IV. DYNAMICS OF SENSITIVITY TO LIGHT

When tadpoles of *Rana clamitans* are subjected to a stimulus of 5 candle-meters, the light being applied from above, the period which elapsed between the time of the application of the stimulus and that of the response had approximately a fixed value. Repeated stimulations at one-minute intervals revealed only slight changes in their period. These slight deviations from the mean of the reaction-time for this and other intensities were only such as were to be expected from normal law of error, as was demonstrated by a study of frequency curves. The reaction time for a set of ninety-eight readings at 5 candle-meters showed an average of 24.1 seconds.

Since not all of the readings were taken during the same day, and since this part of the work was done during the winter months, the variations in temperatures were recorded. Observations based on approximately 2,000 readings with various intensities of light showed no evidence that a temperature variation between 16°C. and 19°C. has an observable effect on the reaction-time. This is not surprising in view of the fact that the velocity of reaction in photic operations is dependent upon the actual amount of light absorbed per unit of time and this amount

varies very little with the rise and fall of temperature. Notwithstanding this fact, after some preliminary work, the temperature during the process of experimentation was regulated to approximately 19°C.

With a constant intensity of 10 candle-meters, the reaction-time was considerably reduced. From the general course of the readings it became evident that the stronger light was more effective, for the animals responded to it with a greater degree of regularity. The reaction-time, which was 24.1 seconds at 5 candle-meters, being in each case the average of about 90 readings, was now reduced to 12.1 seconds. This definite relation between the strength of stimulus and the effect produced was retained with an intensity of 30 candle-meters. The average reaction-time with this intensity for ninety-three readings was 6.1 seconds. The actual values obtained with these three intensities are given in table 1 with their average reaction-times, standard deviation, and probable error. From an inspection of the table, it is evident that a stronger stimulus requires less time to produce the same effect, namely, a simple swimming movement, than a weaker one.

In addition to the values obtained with 5, 10, and 30 candle-meters other intensities were used. These were 0.3, 0.7, 1.2, 15, and 20 candle-meters and with each intensity about 100 readings were secured. In all the experiments in which the relation of the reaction-time to the intensity was studied, this relation between stimulus and effect produced was retained. The weaker the light, the longer was the period required to produce a reaction. With the higher intensities more uniformity of responses was obtained, showing that the energy is more effective as a stimulus, judging from the gradual decrease in the standard deviation and probable error (table 1). The probable errors with the more effective intensities are small. In order to determine whether the differences between the average reaction-times for the various intensities are of sufficient magnitude for statistical value, the probable errors of these differences were computed on the basis that the probable error of the difference

TABLE 1

Reaction-times in seconds of tadpoles of Rana clamitans to lights of different intensities. Temperature, 16° to 19°C. Source of light above

	INTENSITY OF LIGHT IN CANDLE-METERS															
	0.3		0.7		1.2		5		10		15		20		30	
No effect	267	195	45	125	29	24	12	7	5	7	3	8	5	4		
	227	362	46	252	31	18	9	13	6	7	3	5	5	6		
	135	74	195	90	24	20	15	16	5	7	6	3	5	6		
	81	219	107	70	26	24	16	17	8	7	5	5	8	10		
	390	225	61	45	16	20	15	6	6	6	4	4	8	8		
	76	140	185	136	20	10	11	12	16	6	6	7	6	10		
	135	333	83	35	20	18	20	19	14	16	13	8	6	5		
	136	216	197	95	21	25	16	9	7	8	9	4	6	7		
	107	190	75	110	20	34	18	11	10	4	10	5	5	9		
	110	92	83	85	45	15	9	10	14	4	11	9	4	5		
	160	182	107	110	22	24	16	8	10	15	5	3	3	8		
	321	245	134	85	34	16	10	9	6	16	16	10	4	5		
	89	130	231	67	30	12	18	12	6	3	6	6	10	5		
	230	95	66	55	21	20	10	11	5	5	6	3	5	7		
	157	120	127	98	35	33	13	6	15	9	14	9	5	5		
	91	220	126	56	42	25	8	8	11	6	13	6	5	5		
	170	147	60	62	23	25	8	16	17	7	7	6	5	3		
	290	336	102	50	8	25	17	15	7	12	6	5	4	2		
	175	80	104	68	22	26	14	10	10	4	9	6	6	5		
	242	112	100	62	23	13	9	12	13	10	9	6	9	3		
	306	130	271	60	20	36	12	16	12	6	9	12	8	9		
	382	50	105	145	25	9	6	14	9	5	7	11	9	5		
	164	217	55	54	15	27	17	9	5	18	7	5	6	6		
	200	155	99	85	38	16	7	17	5	7	15	5	4	6		
	110	135	113	97	31	20	11	11	8	9	4	10	7	10		
	340	195	48	48	25	22	17	16	12	11	7	6	4	7		
	155	115	44	90	13	14	18	10	4	4	14	6	4	11		
	124	145	54	87	45	9	6	17	4	4	8	5	9	2		
	105	120	69	60	29	9	14	6	5	6	7	12	2	5		
	198	357	158	89	26	24	7	10	5	10	6	5	6	7		
	60	82	55	95	19	13	13	8	10	15	7	10	5	9		
	95	60	137	110	51	22	13	11	10	9	9	6	3	11		
	135	141	95	44	34	22	6	16	12	3	6	7	7	5		
	70	421	45	55	20	46	9	10	6	8	5	5	6	2		
	60	205	72	45	29	13	6	8	6	6	5	5	8	4		
	142	181	39	100	31	18	12	12	11	5	11	5	8	5		
	171	134	60	105	24	39	7	7	8	5	10	17	3	6		
	197	198	110	115	26	14	17	10	10	10	5	6	10	4		
	97	71	47	78	16	30	12	25	11	8	15	13	4	6		
	114	210	55	122	20	63	10	19	3	10	10	8	8	9		

TABLE 1—Continued

	INTENSITY OF LIGHT IN CANDLE-METERS														
	0.3		0.7		1.2		5		10		15		20		30
No effect		153	145	90	119	20	14	9	25	10	11	15	8	5	8
		121	426	105	112	21	55	10	17	9	10	8	7	6	7
		130	162	108	55	30	9	11	10	10	14	6	6	11	3
		159	180	60	141	14	22	11	15	9	13	6	5	8	11
		205				18	35	13	11	10	8	5	5	8	9
		45				24	33	11	12	12	9	6	6	4	7
						14	34	20	9	9	5	13	4	4	
						28	9	11	13	4		12			
						41		10	9	15		15			
						33									
Average.....		173.06		93.10		24.14		12.14		8.57		7.62		6.16	
Standard deviation		87.91		45.93		10.38		4.14		3.62		3.36		2.30	
Probable error of mean.....		6.29		3.32		0.711		0.284		0.251		0.233		0.162	

between the constants of two uncorrelated series is

$$\sqrt{E_1^2 + E_2^2},$$

where E_1 and E_2 are the constants of the two series to be compared. By this means it was found that the probable error of the difference between the means of the reaction-times at 5 and 10 candle-meters is 0.74. Since the difference between the average reaction times at these two intensities is 12, which is more than 16 times the probable error 0.74, and since a similar condition is true for the other higher and more effective intensities of illuminations, it is obvious that the values obtained are of significance in a discussion of the photodynamic nature of the responses.

Between 0.71 candle-meter and 0.3 candle-meter, intermediate intensities were used which do not appear in any of the tables, but it was noticed that in the lower range a point was reached where the animal no longer responded to the stimulus. This point in tadpoles of *Rana clamitans* was found to be 0.3 candle-meter. This with the other facts presented leads to the conclusion that in this animal there is an optimum intensity to

light for the most prompt form of response. As the light intensities fall below this optimum, the effectiveness of the stimulus diminishes to cease at a certain point regardless of the length of time of exposure. Within the range of effectiveness, however, the principle of transference of energy was found to hold, for the action of light upon the photoreceptors produced an effect. This effect was equivalent to the amount of energy received, and it was measured in terms of the time necessary at a definite intensity to produce a response.

From the data thus far given, we have no way of studying the exact changes which occur in the sense organs. It seems obvious, however, that light must be absorbed in order to act as a stimulus and that some energy must be used up in order to initiate a change in the receptors. This in fact is a law governing photochemical phenomena. This law was first demonstrated by Grotthus (1819). It was later confirmed by others (Draper, '41). Lasareff ('07) showed that the Grotthus law is a quantitative photochemical law and that there is a definite relation between the amount of light absorbed per unit of time and the velocity of chemical change produced.

In the light of the observations made thus far and on a basis of our knowledge of the mechanism of other types of receptors better known to us, we are forced to assume that the changes which occur in the sense organs in tadpoles of *Rana clamitans* during illumination are of a chemical kind. The reason for this assumption will become clearer when additional data are presented.

For a discussion, then, of the dynamic process in the photoreceptors we must consider more critically the relation of the reaction-time to the intensity of light. On the supposition that the relation is dependent on the velocity of a chemical change and assuming the validity of the Bunsen-Roscoe law, the product of these two variables should be a constant quantity for all intensities. This leads us to a discussion of table 2, which contains a summary of the results entered in table 1. In table 2 each figure in the second column represents the average of about ninety readings, and in the third column are given the intensity-reaction-time products.

From the apparent nature of the process during illumination, the reaction-time in the table represents a duration of action and the intensity represents the amount of energy which controls the velocity of change in the receptors concerned in the responses to light. In order that a swimming movement be produced, it appears that a definite material change must be produced in the sense organs as a stimulus. That the products of the intensity of light and the time of action agree well with each other for all intensities, can be little questioned from the numerical values entered in the third column. It will be noticed, however, that with the 20- and 30-candle-meter intensities, this

TABLE 2

Summary of results of the average reaction-times in seconds of tadpoles of Rana clamitans to lights of eight intensities and the corresponding intensity \times reaction-time products

LIGHT INTENSITY IN CANDLE-METERS	REACTION TIME	$I \times T$
0.3	No effect	
0.7	173.0	121.1
1.2	93.1	111.7
5.0	24.1	120.5
10.0	12.1	121.0
15.0	8.5	127.5
20.0	7.6	152.0
30.0	6.1	183.0

product is somewhat large. As a matter of fact, this change in the intensity-reaction-time product is not abrupt, but follows a definite course. It is characterized by a gradual increase with the increase in the degree of stimulation. These products with the first five intensities are well within the range of the Bunsen-Roscoe law. The deviation, which becomes so pronounced after an intensity of illumination of 20 candle-meters is reached, occurs in other experiments in similar manner, and will be discussed later in more detail.

When we proceed to examine the results obtained and to plot graphically the values of the intensities on the abscissa and the values of the corresponding reaction-times on the ordinates, a

smooth curve is obtained (fig. 2). This curve is hyperbolic (curve A) and indicates that as one of the variables increases, the other decreases—in this case, according to a constancy, a relation which is retained throughout the whole series of experiments. By the method of least squares from

$$(x - a)(y - b) = C,$$

computing for three constants and employing the summary

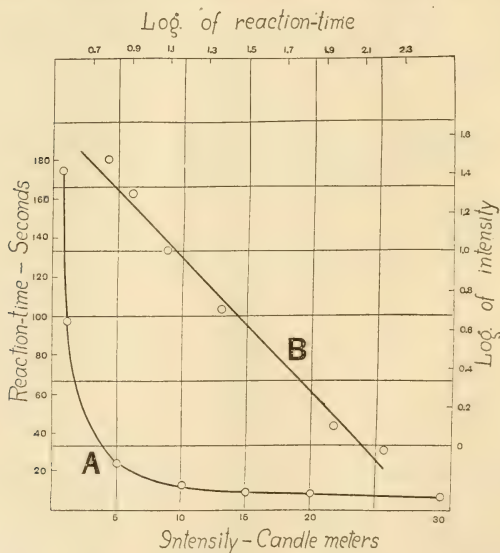


Fig. 2 Curve A illustrates the data in table 2 and represents the relation between the intensity of light and reaction-time. Curve B gives the logarithmic relation between the intensity of light and reaction-time of the same data.

results in table 2, it was found that the data can be represented by the formula:

$$(x - 0.15)(y - 2.85) = 94.48 \text{ or}$$

$$y = \frac{94.48}{x - 0.15} + 2.85$$

Henri and Henri ('12) carried out a series of studies on the effect of ultraviolet rays upon cyclops. From their data it appears that in producing a reflex, the intensity-reaction-time products do not give a constant, but that the hyperbolic curve obtained can be expressed by the formula:

$$y = \frac{a}{x^2} + k.$$

Hecht ('18, '19, '20), in studying the photosensitivity of Ciona and Mya to different intensities of light emitted from an electric bulb, reports that the products of the sensitization periods and corresponding intensities agree well with each other for all intensities.

The Bunsen-Roscoe ('62) law was applied originally to the influence of light on silver chloride. In the responses of tadpoles, there is a striking parallelism between that which has been demonstrated to be true for a purely chemical phenomenon 'in vitro' and the changes occurring 'in vivo,' namely, in the receptors of the tadpoles.

The Bunsen-Roscoe law may be expressed:

$$(1) RT \times I = K,$$

where RT is the time of action, I , the intensity of the light, and K , the constant effect produced. Equation (1) may be put in the following forms:

$$(2) RT = \frac{K}{I}$$

$$(3) \log RT = \log K - \log I,$$

$$(4) \log RT = - \log I.$$

In the last form of the equation (4) the logarithm of the reaction-time is plotted as function of the logarithm of the corresponding intensity, and if the data in table 2 are plotted in accordance with this form, the points give an approximately straight line (B), thus indicating that in the production of a constant effect the velocity of change in the receptors proceeds at a uniform rate. With the gradual increase in the intensities of illumination there is an acceleration in the rate of the changes in the sense organs.

V. PHOTOSENSITIVITY OF THE SKIN AS COMPARED WITH THAT OF THE EYE

1. *The skin*

In terms of the Bunsen-Roscoe law, which was demonstrated as being valid in the photosensitivity of *R. clamitans* tadpoles, it is easy to see that the sensitivity of one animal as compared with another, or of two types of photoreceptors in the same animal, is dependent upon the rapidity with which changes of sufficient strength are produced in the sense organs. Photoreceptors, therefore, are comparable to photographic plates, whose relative sensitivity is determined by the intensity of light and time of exposure necessary to produce the same degree of change on the various plates. In this the Bunsen-Roscoe law finds its first applicability in making possible the classification of degrees of sensitivity in terms of their corresponding intensity-reaction-time products.

When tadpoles with eye and skin intact were subjected to light, it was at first supposed that the responses were those due to stimuli received through the eye. The next problem that naturally suggested itself was to determine the sensitivity of the skin as compared with that of the eye.

In reviewing the extensive literature on the responses of amphibians to light, it is found to be the general opinion that responses may be brought about by stimulating either the eye or the skin. Among some of the workers advocating this view are Graber ('84), Dubois ('90), Parker ('03), Reese ('06), Pearse ('10), Laurens ('14), and more recently Cole and Dean ('17). All of this work has been purely qualitative and in no case has an attempt been made to determine quantitatively the amount of energy necessary to stimulate, 1) the skin alone, 2) the eye alone, and, 3) both eye and skin. The only exception to this is found in the observations of Reese and of Pearse, that when the eyes of *Necturus* were removed, a greater period elapsed before orientation than normally. The short reaction-time that occurs when the eyes are present is ascribed by these workers to stimulations received through those organs.

The eyes of tadpoles of *Rana clamitans* were removed by cutting the skin around them very carefully with a small sharp dissecting knife and then placing curved scissors under the eyeball in such a way that all muscles and nerves were cut with one stroke and the eye removed. Such tadpoles were allowed a few days in which to recover before being subject to experimentation.

When the work with blind tadpoles was begun, it was intended to use the same number and range of intensities as were used for testing the photosensitivity of tadpoles with eyes and skin. Very early in the work, however, it became evident that the reaction-time of blind tadpoles was the same as that of tadpoles with eyes and skin illuminated. When blind animals were repeatedly stimulated with light of 1.2-candle-meter intensity, the individual readings corresponded very closely with those obtained from the animals with eyes and skin stimulated. Similarly, it was shown that both in tadpoles without eyes and in tadpoles with eyes and skin, the same period elapses between the stimulation and response under the influence of 15 candle-meters. Table 3 gives the actual observations at these two intensities, as well as the probable error, standard deviation, and the intensity-time products. These products agree well with each other, and likewise with those of corresponding intensities when eye and skin were illuminated.

These surprising results suggested that with light falling vertically upon laterally placed eyes, such as those in tadpoles, the eyes may not have received the full stimulus applied and hence the similarity of results. For this reason the experiments with other intensities were not carried further in the manner described, but the subsequent tests and apparatus were modified so that the stimulus was applied from the side.

The individuals to be tested were placed in a rectangular glass aquarium 38 cm. long and 10 cm. wide (fig. 3, *m*). By a tightly fitting plate this width was subdivided longitudinally into two compartments, one of which was just wide enough to allow freedom of movement of animals forward and backward, but always parallel to the side of the aquarium. At a distance in

front of the aquarium sufficient to bring about the desired intensity of illumination was placed the electric bulb (*n*) properly connected with a switch within easy reach. The arrangement of the remainder of the apparatus was the same as when the light came from above.

TABLE 3

Reaction-times in seconds of eyeless tadpoles of Rana clamitans to lights of different intensities. Temperature, 19°C. Source of light above

INTENSITY OF LIGHT IN CANDLE-METERS										
1.2					15					
127	42	135	117	168	5	13	3	11	9	
96	95	61	196	65	5	13	7	11	16	
144	99	49	173	55	8	5	7	6	9	
97	71	87	101	98	6	9	4	5	7	
85	40	134	92	48	7	8	8	6	9	
67	85	45	156	60	18	8	5	13	5	
93	104	91	171	154	15	9	6	8	7	
73	96	202	71	101	15	4	4	5	6	
102	75	47	104	44	5	7	4	12	11	
45	141	86	136	143	5	4	5	11	14	
103	105	188	50	67	5	6	5	7	9	
240	55	90	111	71	5	5	4	11	11	
119	290	115	58	68	8	11	10	6	5	
180	65	119	151	55	9	7	5	10	10	
180	81	47	64	116	9	5	3	10	9	
47	233	97	45	56	9	5	4	18		
45	72	105	116	167	11	16	5	8		
			159	258	5	12	7	7		
				132	8	4	5	8		
					9	10	5	10		
Average.....					7.9					
Standard deviation.....					3.38					
Probable error of mean.....					0.24					
Intensity \times reaction-time.....					119.1					

Usually two individuals at a time were placed in the aquarium. These were kept far enough apart to avoid any shadow effect. After the application of the stimulus the time of reaction of the individual to move first was recorded. With an apparatus thus arranged the eye as well as the side of the animal was illuminated.

The sensitivity of animals was tested under the two conditions: 1) skin alone and, 2) eye and skin. Such experiments furnished further data for a test of the validity of the Bunsen-Roscoe law as applying to the photosensitivity of this animal.

In table 4 are given the reaction-times of tadpoles to lights of different intensities when eye and skin are exposed to light (A) and after the eyes had been removed (B), the light in both instances being applied from the side. The reaction-times of

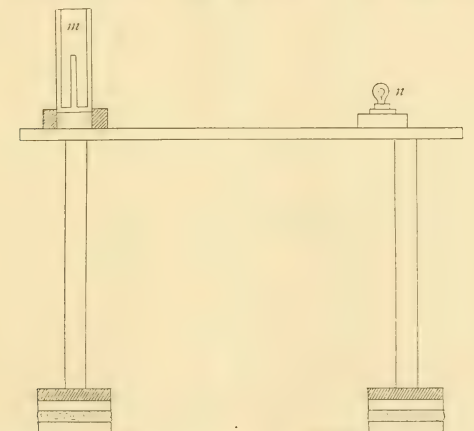


Fig. 3 Diagram of apparatus for testing the sensitivity of the eye as compared with the skin. *m*, rectangular glass aquarium; *n*, light.

animals with eye and skin were so similar to those obtained from corresponding intensities with blind tadpoles either when the source of stimulus was from the side or from above, that it was not found necessary to carry the work beyond five intensities, only four of which were effective. This is apparent by comparing the results in table 4 with those in tables 1 and 3. That with the gradual increase in the intensity of illumination the stimulus becomes more and more effective for bringing about a prompt form of response is again demonstrated here by the gradual decrease in the standard deviation and probable error

TABLE 4

Reaction-times in seconds of tadpoles of Rana clamitans to lights of different intensities: A, with eye and skin exposed to light; B, with skin only exposed to light. Light applied from the side

		INTENSITY OF LIGHT IN CANDLE-METERS				
		0.3	5	10	15	20
A. With eye and skin exposed.....	No effect	39 21	11 9	8 9		
		16 43	18 23	8 8		
		22 28	11 16	14 11		
		23 22	8 12	8 8		
		18 35	15 6	5 10		
		46 13	8 10	10 4		
		11 28	14 22	10 4		
		44 6	11 13	14 8		
		34 24	13 15	10 8		
		45 37	15 12	9 9		
		13 42	14 6	8 13		
		23 10	7 9	14 10		
		11 6	12 16	9 6		
		10 54	10 15	9 12		
		24 16	12 12	7 7		
		43 6	12 13	8 7		
		14 7	8 11	6 8		
		13 15	9 20	7 6		
		19 12	9 12	5 11		
		10 19	10 13	5 5		
		13 29	8 8	8 10		
		14 35	8 20	11 9		
		15	16 15	4		
		17	10	7		
				5		
Average.....		22.61	12.28	8.34		
Standard deviation.....		12.70	3.94	2.57		
Probable error of mean.....		1.28	0.39	0.26		
Intensity \times reaction-time.....		113.0	122.8	125.1		

accompanying the increase of the intensity of illumination. The intensity-time products agree well with each other under the two conditions and with those recorded in table 2. With a light intensity of 20 candle-meters the characteristic deviation already mentioned again becomes evident.

TABLE 4—Continued

	INTENSITY OF LIGHT IN CANDLE-METERS				
	0.3	5	10	15	20
B. With skin only exposed.....	No effect	26 14		10 13	
		20 15	17 4		
		24 35	11 9	12 5	5 10
		23 35	13 9	8 5	5 6
		21 16	15 10	7 5	3 3
		16 11	8 14	16 6	9 8
		29 20	10 7	8 10	6 7
		30 31	9 13	17 11	5 12
		21 30	12 11	6 14	9 11
		11 19	13 16	9 4	6 12
		32 16	9 18	4 7	5 14
		20 40	24 5	5 9	5 8
		12 25	28 6	8 9	4 4
		21 40	14 9	9 9	3 5
		20 13	16 23	10 9	5 3
		25 40	5 8	4 11	8 4
		32 45	18 8	11 3	7 8
		27 18	16 14	5 14	7 10
		21 17	21 11	4 12	9 9
		19 22	21 9	6 9	14 14
		35 10	16 22	5 4	5 8
		25 24	4 12	5 10	4 9
		25 32	20 20	9 16	10 8
		29 15	15 7	4 17	6
		23 21	23 7	8	6
Average.....		23.82	13.25	8.51	7.15
Standard deviation.....		8.31	6.03	3.78	2.38
Probable error of mean.....		0.80	0.59	0.38	0.24
Intensity \times reaction-time.....		119.1	132.5	127.6	143.0

In figure 4 are plotted the data with blind tadpoles as recorded in table 4. In order to secure a wider range of comparison to this was added the results with 1.2 candle-meters in table 3. When the average reaction-times are plotted on the ordinates as functions of the intensity of light, the plotted points give a hyperbola (curve A). The logarithm of the average reaction-times plotted as function of the logarithm of the intensity, gives a straight line (B). From the hyperbolic representation it is clear that the reaction time holds a definite relation to the inten-

sity and that the relation is inverse. Curve B characterizes the steady rate of increase in the velocity of change in the receptors with an increase of intensity of illumination. These graphic representations and the intensity-reaction-time products furnish additional evidence that the changes in the photoreceptors in this animal proceed according to the Bunsen-Roscoe law.

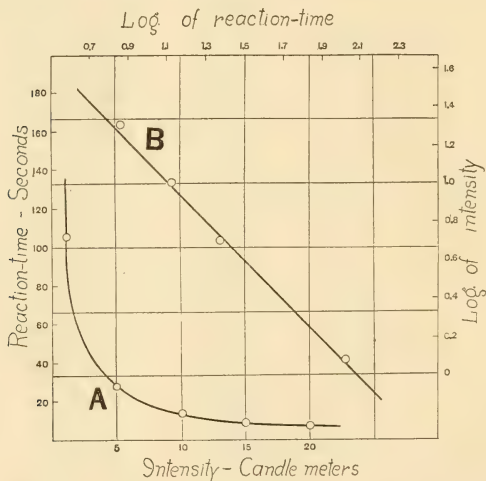


Fig. 4 Curve A illustrates the data in table 4. The reaction-time is plotted on the ordinates, as function of intensity, plotted on the abscissa. Curve B represents the logarithmic relation between the reaction-time and intensity of illumination of the same data.

If, now, the curves in figure 2, illustrating the relation of the intensity of illumination to the reaction-time of tadpoles with eye and skin, and the curves in figure 4, giving this relation of records obtained from blind tadpoles, are plotted with regard to the same axis, they will be seen to coincide almost exactly. This is apparent from an inspection of the data and curves, and for this reason no such graphic comparison has been undertaken.

2. The eye

From the recorded data and graphic representations it appears that when the eyes and skin of tadpoles are illuminated, the responses are those due to stimuli received through the skin. To test this further, rays of known intensity were concentrated upon the eye only. To avoid stimulation of other parts of the body, the following method was employed. A box measuring

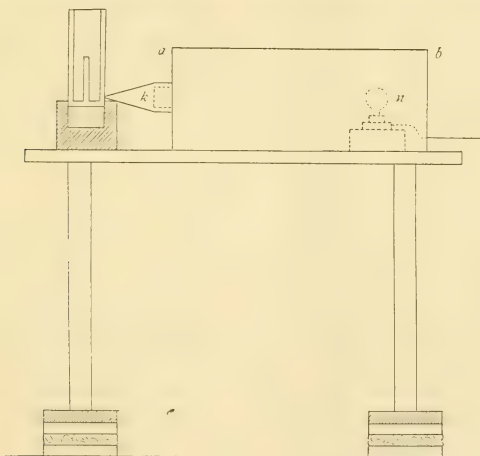


Fig. 5 Diagram of apparatus for testing the sensitivity of the eye. *a, b*, a box constructed of heavy black cardboard; *k*, tightly fitting hollow cone; *n*, light.

28 cm. in length and 9 cm. in height and in width was constructed of heavy black cardboard (fig. 5, *a, b*). One end of the box was so modified by a cylindrical projection as to receive a tightly fitting hollow cone (*K*) terminating in a small opening, slightly less than the diameter of the eye of the tadpole. A light bulb (*n*) of 0.3 candlepower was enclosed in the box and the distance from the end of the cone was so adjusted as to give a light intensity of 5 candle-meters at the position of the animal, this being the intensity which proved effective with the experi-

ments on eye and skin and on skin alone. The rest of the apparatus was the same as shown in figure 3. With the aid of a dim light, the end of the cone was closely applied in the direction of the eye lying next to the side of the aquarium, so that when the light was turned on, the rays reached the eye only. Such an arrangement made it possible to illuminate the eye by an accurately measured intensity of light.

The sensitivity of the eye was studied in detail. Several individuals, which had previously responded with a great degree of regularity, were used. Repeated stimulations of the eye at one-minute intervals produced no response. If, however, the cone was slightly rotated or its position moved forward or back in the process of adjustment, occasional responses were observed. Such reactions were attributed to the stimulus of motion rather than to the influence of light, for the same degree of stimulus accompanied with no motion led to no response.

In the eye of the tadpoles, however, we find an apparatus adapted for receiving light stimulus. It is not to be questioned that during illumination chemical changes are occurring in the retina. In fact, if we consider the electrical changes in the retina due to light of a photochemical order, as demonstrated by Dewar and McKendrick ('74), Waller ('00), Einthoven and Jolly ('08), we have a chemical change in the retina in a given time dependent upon the intensity of illumination and measured by the amount of electrical current produced. This has been demonstrated by all the workers on retinal changes. The lack of responsiveness of *Rana clamitans* tadpoles when the stimulus is applied only to the eye and the orderly responses when the skin is stimulated becomes clear when we consider the difference in specialization and organization of these two types of receptors. The skin receptors are connected with the lower reflex centers, while the eye is a more specialized analyzer for image-forming functions. The distinction in function is made clear by Pavlov, who pointed out that in the lower centers the reflexes are unconditional; that is, knowing the nature of the stimulus, the responses can be predicted. When the stimulus is applied to a higher, more specialized sense organ, the occurrence or a

visible response is dependent on other conditional factors. Again the failure of the animals to produce a reflex when the eyes are illuminated may be due to the inaccuracy of the visual apparatus. An example of this is found in the mollusk *Pecten*. In *Pecten* along the free edge of the mantle are situated a number of eyes, each one histologically representing an elaborate visual apparatus. Patten ('87) found in the eyes of *Pecten* a true cornea, lens, ciliary muscles, retinal structures, and nerve fibers, all occupying the same position as in the eye of the higher vertebrates. Uexküll ('12) reports that even the image of an enemy, such as a starfish, is not sufficient to initiate a response in *Pecten*, unless the image formed in the retina is reinforced by a movement of the object. Similar observations on *Pecten* have been recorded by Wenrich ('16).

The experimental evidence, therefore, under the three conditions: 1) eye and skin, 2) skin alone, and 3) eye alone, in terms of the amount of energy necessary to act as a stimulus as exhibited by the intensity-reaction-time products, shows that this amount of energy is constant. Repeated stimulations of the eye with intensities which were effective when the photoreceptors of the skin were illuminated, led to no responses. It appears therefore, that, under the conditions of the experiments, the tadpoles of *Rana clamitans* are stimulated through the skin and that the eyes take no part in the recorded responses to light.

VI. VELOCITY OF CHANGES IN THE RECEPTORS WITH HIGHER LIGHT INTENSITIES AND THE DURATION OF SECONDARY PROCESSES FOLLOWING SENSITIZATION

The steady increase in the rate of changes in the photoreceptors with the gradual increase in the intensity of illumination was demonstrated graphically when the logarithm of the intensity of light was plotted as function of the corresponding reaction-times. This relation was found to be linear (figs. 2 and 4). The more exact relation between the velocity of change in the sense organs and the dependence of this on the intensity factor, as well as the exact range within which this relation holds true, can be studied by an employment of the reciprocity law as derived from the Bunsen-Roscoe law formula.

The Bunsen-Roscoe law can be expressed:

$$(1) I \times T = K,$$

where I stands for the intensity of light, T indicates the reaction-time, and K the constant effect produced. By transposing, this equation can be written:

$$(2) I = \frac{K}{T} \text{ or } \frac{1}{T}$$

where the constant effect, K , produced has been considered as unity. In this form we have a reciprocal relation and for practical purposes it can be interpreted to mean that the constant amount of work done divided by T , the time in which the work is done, expresses the velocity of change or the rapidity with which this is done. The greater T becomes, the slower becomes the velocity of change, and this whole relation from the nature of the equation is dependent upon the intensity of light.

A graphic representation, therefore, of the data secured under the conditions: 1) sensitivity of eye and skin and, 2) skin alone, when the reciprocal of the reaction-time is plotted as function of the intensity (equation 2) produces a curve which illustrates the velocity of chemical change. This is shown in figure 6, where for curve A the data in table 2 were employed; for curve B the average reaction-times of blind tadpoles as shown in table 4 were used in addition to the results obtained with 1.2 candle-meters as recorded in table 3.

It is obvious from the two curves, that the velocity of change in the receptors is approximately linear up to 15 candle-meters, above which a gradual deviation occurs. This has already been observed in the tables on intensity-reaction-time products in three sets of readings, under three different conditions (tables 1, 2, 3).

In reflex actions due to light, Hecht ('18) observed in *Ciona intestinalis* a delay in the response after sensitization during which the animal need not be illuminated in order to respond. This secondary period he has termed the 'latent period,' and for *Ciona* he has assigned to it a value of 1.7 seconds. In subsequent researches Hecht ('19 a, b) has demonstrated the striking

phenomenon that the actual sensitization period is extremely brief, while the time occupied by secondary processes may vary between one and four seconds, depending upon the duration of illumination and temperature. Henri et Henri ('12), in studying the excitability of *Cyclops* by ultraviolet light, find that the animal responds in 0.2 to 0.1 second after cessation of illumi-

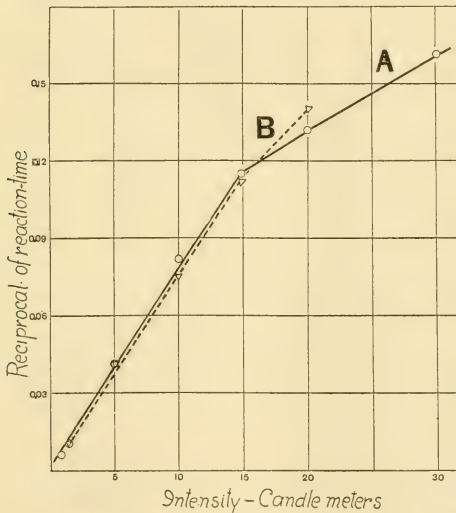


Fig. 6 Curve A represents the relation of the reciprocals of the average reaction-times plotted as function of intensity when eye and skin are illuminated from above. Curve B shows the relation of the reciprocals of the reaction-times plotted as function of intensity when blind tadpoles are illuminated from the side.

nation. Since only the duration of illumination represents the actual time occupied for the necessary photochemical processes in the peripheral organs, the deviation in the reciprocal curves in figure 6 may be regarded as indicative of a secondary process in the sense organs, nervous system, and muscles, which as yet has not been analyzed in the study of the light reactions of tadpoles.

For, as the intensity factor increases and the reaction-time becomes short, a secondary period, for secondary processes after sensitization, of appreciable value may cause an increase in the intensity-reaction-time products. From another standpoint it may be claimed that in the lower range of intensities the Bunsen-Roscoe law holds good, but with higher illumination the velocity of changes taking place in the receptors is no longer proportional to the intensity factor. In such a case a proportionally longer period is required to produce the amount of stimulating substance necessary in order to activate the nerve endings.

In searching for the duration of a period occupied by secondary processes after sensitization, during which the animal need not be illuminated in order to respond to the stimulus, the following method was employed. By increasing the intensity of light, the reaction-time was reduced to approximately three seconds. It was found possible to reduce further the reaction-time by employing still higher degrees of stimulation. Thus the average value obtained with 200 candle-meters was found to be 1.7 seconds and with 500 candle-meters 0.76 second. With higher intensities than 500 candle-meters, it was found difficult to measure accurately with a stop-watch the reaction-time, because of its extreme shortness. The actual values obtained with 60, 200, and 500 candle-meters are given in table 5.

If the reaction-time is composed of two periods, a sensitization period and a period for other processes following sensitization, then from the reaction-time with 500 candle-meters it is seen that 0.76 second must represent both these processes. From this it can be concluded that the duration of this secondary period must be very short and that, whatever its value is, it must lie at or below 0.76 second. If the deviation noted with the higher intensities in the intensity-reaction-time products is due to such a period, then a value lying between 0 and 0.76 second ought to be found which when subtracted from the average reaction-times for the various intensities would yield results capable of right-line plotting. That this is not the case is seen from figure 7, in which the curves were plotted by the same method as was employed in figure 6, except that from average

reaction-times was subtracted 0.7 second, an assumed value for the probable duration of secondary processes. The general course of the curves in figure 7 is the same as that shown in figure 6, and it is characterized by the same deviation in the higher intensities. If, now, the values obtained with 60, 200, and 500 candle-meters are also plotted, the region in the curves representing these intensities assumes an irregular form. Other,

TABLE 5

Reaction-times in seconds of blinded Rana clamitans tadpoles to light intensities of 60, 200, and 500 candle-meters. Source of light above

	INTENSITY OF LIGHT IN CANDLE-METERS		
	60	200	500
	2.3 3.0	1.4 1.4	0.7 0.7
	2.5 3.0	1.8 1.2	1.0 0.7
	3.0 2.9	2.0 1.4	0.7 1.6
	2.3 3.2	1.4 1.4	0.6 0.8
	2.4 (over time)	2.4 1.6	0.8 0.8
	3.0 (over time)	2.0 1.4	0.8 2.4
	3.1 3.0	3.0 1.6	0.8 0.6
	3.2 3.0	1.6 2.0	2.0 0.6
	3.1 2.9	2.0 1.4	0.6 0.6
	(over time) 4.0	1.6 1.8	0.8 1.0
	3.0 3.2	1.8 1.6	1.8 0.6
	3.1 3.1	(2 over time)	0.8 0.8
		1.6	1.8 0.8
			0.6 0.8
			0.7
Average	2.9	1.7	0.76

smaller, assumed values were subtracted from the average of the reaction-times and curves for these values were also plotted. They were 0.2 second and 0.5 second. Such a treatment of the data led to no improvement in the reciprocal curves. With an assumed value of 0.5 second for secondary processes there was a slight improvement in the intensity-time products. But here also the gradual characteristic deviation with the higher illuminations was retained. This becomes clearer from an inspection of table 6. The deviation with the higher intensities

is, therefore, gradual and it seems to proceed with an approximate constancy.

The photodynamic nature of *Rana clamitans* tadpoles, therefore, differs from that of *Ciona* (Hecht, '18) and of *Mya* (Hecht, '19 a, b, '20) in that nearly all of the reaction-time in the tad-

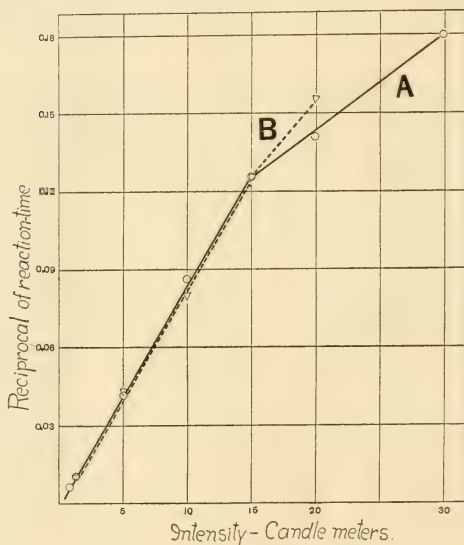


Fig. 7 Curve A represents the relation of the reciprocals of reaction-times of tadpoles with eye and skin, minus a probable secondary period of 0.7 second, plotted on the ordinates, as function of intensity, plotted on the abscissa. Curve B, reciprocals of reaction-times of blind tadpoles, minus 0.7 second, plotted on the ordinates as function of intensity of light.

poles represents a sensitization period and that the time occupied for secondary processes seems to be extremely short.

In view of the close agreement between the intensity-reaction-time products up to 20 candle-meters and the consistency of deviation with the higher illuminations, it appears that the Bunsen-Roscoe law holds true only within a certain limited

range. With the higher degrees of stimulation, the dependence of the velocity of change upon the intensity is retained, but there is a slight slowing down in this velocity. This condition is further illustrated by the empirical formula for the curve presented early in this discussion (p. 250). The formula was found to be as follows:

$$y = \frac{94.48}{x - 0.15} + 2.85$$

in which y is the reaction-time in seconds, x , is the light intensity in candle-meters, and 0.15, 2.85 and 94.48 are three con-

TABLE 6

Summary table, in seconds of average reaction-times, of products of reaction-times and light intensities, and of these values modified by subtractions (0.5 second and 0.7 second) for assumed secondary processes

	LIGHT INTENSITY IN CANDLE-METERS									
	0.7	1.2	5	10	15	20	30	60	200	500
Reaction-time.....	173	93.1	24.1	12.1	8.5	7.6	6.1	2.9	1.7	0.76
$I \times T$	121.1	111.7	120.5	121	127.5	152	183	174	340	380
Reaction-time—0.5 sec.	172.5	92.6	23.6	11.6	8.0	7.1	5.6	2.4	1.2	0.26
$I \times T$	120.7	111.1	118	116	120	142	168	144	240	130
Reaction-time—0.7 sec.	172.3	92.4	23.4	11.4	7.8	6.9	5.4	2.2	1.0	0.06
$I \times T$	120.7	110.8	117	114	117	138	162	132	200	30

stants. This formula satisfies well the actual data obtained. The constant a of the original formula (p. 250) is equal to 0.15 in the empirical representation and means that with the lower intensities of light the Bunsen-Roscoe law does not hold true for all values of x , but that there is a certain minimum intensity of light beyond which the light produces no apparent effect upon the organism. This was demonstrated experimentally. The constant b ($= 2.85$) denotes that with the higher intensities and as the degree of illumination approaches infinity, a point is reached where the reaction-times are the same regardless of the intensity of illumination. This point in the reaction-time of *Rana clamitans*, however, is not reached abruptly, but gradually and with a definite constancy.

Hecht ('18, '19 a, b) observed no such deviation as described above. It is to be noticed, however, that the ratio of the range intensities employed by him is about 1:20, while the ratio of range of illuminations employed in the present study covers a field of light intensities 1:500. With a range of intensities 1:20 my results are in accord with those obtained by Hecht.

VII. PHOTSENSORY RECOVERY

After animals which have been kept in the dark are exposed to a continuous illumination of 10 candle-meters for an hour, they show no sensitiveness to light. In terms of the changes taking place in the photoreceptors, this may be said to be due to a reduction of the photochemical substance to a minimum, and the changes produced in the peripheral sense organs thereafter to be of insufficient rate to act effectively as stimuli. As a starting point, this enables us to study the nature of photosensory recovery.

The following method was employed in studying recovery. The animal to be tested was placed in a white porcelain dish and with an overhead light its sensitivity was determined. If the animal responded with regularity, it was exposed to a continuous illumination of 10-candle-meter intensity till no longer sensitive to the light stimulus. The time necessary for this process varied with different individuals from forty to sixty minutes, but for uniformity of procedure it was decided to use one hour of continuous illumination. At the end of that period the animal was tested several times, and if no response was noted within two minutes from the time of the application of the stimulus, the animal was left in the dark ten minutes and its sensitivity again tested. After ten more minutes in the dark its reaction-time was again tried. Four such experiments were made with four animals, the summary results of which are given in table 7.

From an inspection of the table it can be seen that in the course of photosensory recovery, the reaction-time at first is long, then gradually decreases, and finally becomes a constant quantity. After this point, repeated stimulations at ten-minute intervals in the dark revealed no very marked increase

or decrease in the reaction-time. This period is reached in fifty minutes. The condition is shown graphically in figure 8, where the data in table 7 are used, the reaction-time being plotted as the function of time in the dark. The curve also

TABLE 7

Summary table of average reaction-times in seconds for photosensory recovery in four tadpoles of Rana clamitans

TIME IN DARK	REACTION-TIME
<i>minutes</i>	<i>seconds</i>
10	52.3
20	30.0
30	20.6
40	15.2
50	12.8
60	12.7

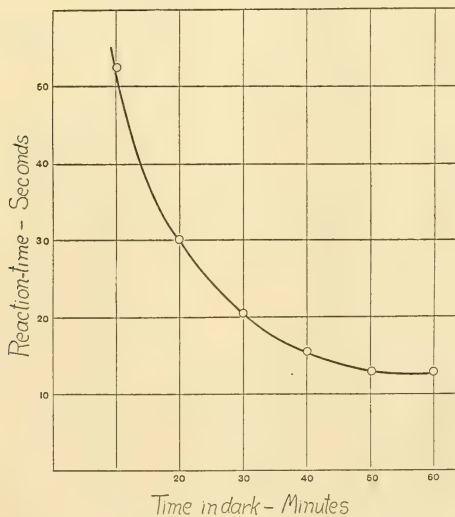


Fig. 8 Curve illustrating the process of photosensory recovery. The reaction-time (seconds) is plotted on the ordinates as function of time in the dark (minutes) on the abscissa.

illustrates the velocity of the process of recovery and the nature of the chemical changes in the receptors. In the beginning the process moves with extreme rapidity. The velocity of change in the sense organs then gradually decreases in rate, as seen in the diminution of the slope in the curve, and finally the curve in its lower course is almost parallel to the abscissa, thus indicating that the process of photosensory recovery has reached a state of equilibrium.

From the nature of the experimental results obtained, it is evident that the reaction-time at any moment in the course of recovery is an expression of the amount of substance present in the system at that moment. The relation is inverse, for the greater the amount the shorter is the period required to produce a definite change.

VIII. THE PROCESS OF FATIGUE

When tadpoles are subjected to continuous illumination for a considerable time they become no longer sensitive to light. The course of the process of fatigue was studied, the method employed being similar to that used in studying the process of photosensory recovery. The individuals to be tested were placed in a porcelain dish with the source of illumination above them; whereupon their photosensitivity was tested. Only those tadpoles were used which had been kept previously in the dark and which showed normal behavior. Usually two individuals at a time were placed in the vessel. In such cases only the reaction-time of the individual to respond first was recorded. A light intensity of 10 candle-meters was employed. The individuals were exposed for ten minutes to continuous illumination, and at the end of that period, after one minute of darkness, the light was again applied and the reaction-time of the first individual to move was recorded. After another ten minutes of continuous illumination, the process was repeated. Only one reading was taken after the application of the stimulus. Now and then in the course of the experiments irregularities occurred in the midst of readings which otherwise showed uniformity of response. To check the significance of an occasional deviation another record was taken immediately after the diverse reading,

and the average of these two was considered to represent the true value. Almost without exception, the second reading taken agreed well with the previous uniform results. Five experiments were made, a summary of which is given in table 8. In addition to this, the data are represented graphically in figure 9, the reaction-time being plotted as a function of time

TABLE 8

Summary table of average reaction-times in seconds for tadpoles of Rana clamitans in the process of fatigue. Light intensity, 10 candle-meters

TIME IN LIGHT	REACTION-TIME
minutes	seconds
0	12.0
10	23.4
20	52.3
30	71.0
40	Over time

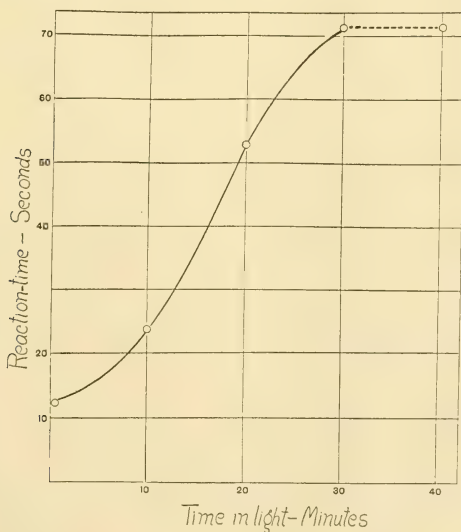


Fig. 9 Curve illustrating the course of the process of fatigue. The reaction-time, in seconds, is plotted as function of time in the light, in minutes.

in the light. From the table it is seen that the reaction-time is at first normal and then gradually becomes longer with the increase of continuous illumination. After thirty minutes, although the individuals were still responsive, they showed a reaction-time suggesting that they were nearing exhaustion. With only one exception, complete fatigue occurred after forty minutes of continuous illumination. From the curve in figure 9 it is seen that the process of fatigue is characterized at first by a slow velocity of change, then by a gradual increase, then by a decrease, and finally by a sudden break, where the animals no longer responded. This is in marked contrast with the velocity of change during photosensory recovery where the rate of change in the receptors at first increases rapidly, then gradually decreases, and finally becomes constant—a process characteristic of monomolecular reaction. The process of fatigue, however, is not a simple process progressing only in one direction, but it is being opposed also by the return of a certain amount of the decomposed material to its original state, at a rate independent of the intensity of illumination. This was shown in the study of photosensory recovery. Because of this fact, therefore, the observed results furnish us with only a limited quantitative criterion for an analysis of the chemical process in the sense organs during illumination,

The dependence of the velocity of change upon the active masses finds close analogy in the retinal electrical changes due to illumination. Dewar and McKendrick ('74) showed that the effect of continuous action of light upon the retina of the frog is marked at first by an increase in the electrical change, then by a gradual diminution, and finally by constancy. Similar phenomena in the electrical changes of the retina have been shown by Kuehne und Steiner ('80), Waller ('00), and Einthoven and Jolly ('08), who demonstrated that, whether the currents are positive or negative, the retina becomes less and less sensitive to the light after it has been subjected to continuous illumination.

Hecht ('18) showed that *Ciona intestinalis* is fatigued by light when the individuals are repeatedly stimulated after one-minute intervals in the dark. *Rana clamitans* tadpoles, re-

peatedly stimulated at one-minute intervals, revealed no substantial change in the reaction-time. The difference in behavior becomes clear when we recall that in *Ciona* the process of recovery requires from two to three hours, while in the tadpoles it is only fifty minutes. From this it is not difficult to see that the more rapidly an animal is able to recover decomposed material in the sense organs the less rapidly will it become fatigued.

IX. DISCUSSION

1. The nature of the process

At various points in this paper it has been stated that the process active in the sense organs of *Rana clamitans* tadpoles during illumination is of a photochemical kind. This conclusion was based on the regularity with which responses took place under measured amount of radiant energy and also upon the definite relation between reaction-time and the amount of active mass during the photosensory recovery and fatigue. That the process in the photic sense organs of animals is of a chemical character rather than of the nature of diffusion or some other physical process finds strong support in a comparative study of temperature coefficients of photochemical reactions as compared with those of physical phenomena. No attempt was made to determine the temperature coefficient of the tadpoles in their responses to light, for preliminary study revealed that the animals placed in water below 10°C. become very active and show numerous spontaneous movements at short intervals, while tadpoles placed in water above 22°C. become sluggish and unresponsive. It is a well-known fact from general chemistry, however, that the velocity of a chemical reaction increases with a rise of temperature. The temperature coefficient of a true chemical process is between 2 and 3 for a change of temperature of 10°C. In chemical changes due to light the temperature coefficient has much lower value than in chemical process proper, and it is found to lie between 1.1 and 1.2. This is evident from the fact that the rate of photochemical change where light is the stimulating agent is dependent upon the amount of light

absorbed (Grotthus, 1819; Draper, '41), and this varies very little with a change of temperature.

Henri et Henri ('12) find that the excitability of *Cyclops* by ultraviolet light is independent of temperature. Hecht ('19), in studying the photodynamic nature of *Mya* with respect to the amount of energy received and the length of the reaction-time, assigns a value for the temperature coefficient of 1.1 for a change of 10°C. These two studies are the only ones to my knowledge on the temperature coefficient of animals responsive to light. The magnitude of the temperature coefficient is in close accord with those of chemical reactions due to light, thus affording strong evidence that the sensitivity of animals to light is of a photochemical kind. Osterhout ('17) points out that a process which has a temperature coefficient as high as 2 cannot be considered of physical nature. For photosynthesis, he assigns a value of 1.7 for the temperature coefficient. This slightly higher value, he holds, is evidence that the process of photosynthesis is of a combined nature, photochemical and chemical.

In the light of the observations made in the study of the photodynamic nature of the responses of *Rana clamitans* tadpoles, namely, that, with a constant intensity of illumination at one-minute intervals in the dark, the reaction-time shows no marked variation from a value which is also constant, we have a suggestion that the changes in the receptors are of an orderly type. Whatever these changes are, they must involve a photochemical substance, normally present in the receptors, a part of which undergoes a change to a different state under the influence of light. For simplicity, at this point of the discussion this may be expressed by assuming a change from A to B, where A is chemically different from B and only in this last state capable of acting as a stimulus for the nerve endings with which it is in immediate contact. Such a hypothetical representation is usually employed for the measurement of photochemical changes in chemistry, and it is variously modified by Weigert ('11) and Sheppard ('14) to meet the requirements of the order of photochemical reactions which they have classified. Two

such alternative photochemical systems have been employed by Hecht ('18, '19a, b, 1920) in a discussion of the nature of the photosensitivity of Ciona and Mya.

2. *The photoreceptors*

The isolation of skin photoreceptors for histological examination has met with difficulty by all workers who have undertaken it. No progress has been made other than regional localization of such organs. It is not difficult, however, to imagine a structural condition for photoreception such as described by Polara ('06). In *Holothuria poli* he describes pigment cells in close association with nerve terminations. These pigment cells, he suggests, are a part of a sensory apparatus for the reception of light. As a result of continuous illumination, the pigment cells are decolorized and associated with this process, there is a loss in the photosensitivity of the animals. In the darkness pigment reappears and with it photosensitivity is regained. Similar observations have been made by Crozier ('14) in *Holothuria surinamensis* and *H. captiva*. A fluorescent pigment, confined to the superficial layer of the integument, showed absorption in the blue-green band of the spectrum, thus suggesting that the process of decolorization is of a chemical nature.

The photoreceptors in the tadpoles must be so situated in the integument as to be partly or entirely concealed when the melanophores are in an expanded condition. Tadpoles dark in appearance either did not respond when light was applied or showed a reaction-time totally different from tadpoles light in appearance. This behavior was studied in detail and with considerable care. Before the facts of this situation were known, much difficulty was experienced in the experimental work. For this reason, after certain preliminary trials, only tadpoles of the same degree of coloration were used.

Photoreceptors in animals are analyzers for appreciating changes in light and darkness. When the animals are living in their natural habitats this type of sense organs must play an appreciable rôle. In species which respond only to a decrease of

light intensity, the appreciation of a shadow may serve as a warning of the approach of an enemy. In species capable of responding to an increase of intensity, the appreciation of the change of illumination must also be of value. It is evident, therefore, that animals better equipped for judging light and darkness and for responding to such stimuli, are better adapted for their life struggle. The more sensitive the receptors are, the more value they must have for the individuals.

In reflexes due to light we recognize two important periods. These are a sensitization period, during which energy is received for the chemical processes necessary in the sense organs, and a secondary period which is occupied by such processes as the diffusion of chemical products to the nerve endings, transmission of the impulse, and a contraction of the muscles of the organ involved in the response. When the time taken up by the secondary processes is considerable, it must be deducted from the reaction-time in determining the actual duration of the preceding chemical process.

In judging the relative photosensitivity of animals, a separation of the reaction-time into its two phases, with the assignment of a value for each, is important. Animals, however, whose photoreceptors are characterized by the quickness with which they can execute the chemical processes necessary for the stimulation of nerve endings, but also by slowness in the secondary processes, gain little from the quickness with which the first process is executed. In such animals the duration of both processes must be considered in judging photosensitivity.

The Bunsen-Roscoe law furnishes us with an important quantitative means in testing the photosensitivity of animals. By extending this type of experimentation to a large number of animals, it will be possible to gain important insight into the nervous mechanism of these forms. The intensity-time products of three such animals have already been recorded: *Ciona intestinalis* (Hecht, '18), *Mya arenaria* (Hecht, '19 a, b, '20), and *Rana clamitans*. It is hoped that this work will be further extended.

X. CONCLUSIONS

1. There is an optimum intensity for the responses in *Rana clamitans* tadpoles to light.

2. At and below 0.3 candle-meter intensity, the light ceases to have a physiological effect, regardless of the time of exposure.

3. With effective light intensities below 20 candle-meters, the changes in the receptors during illumination proceed according to the Bunsen-Roscoe law.

4. With intensities higher than 20 candle-meters, a deviation occurs in the intensity-time products which seem to proceed with a definite constancy.

5. Nearly the whole of the reaction-time in *Rana clamitans* represents a sensitization period.

6. The eyes are not necessary for the responses of tadpoles to light of the kind used in these experiments.

7. Tadpoles subjected to continuous illumination of definite duration become no longer sensitive to light.

8. In the process of fatigue, the reaction-time at any moment has a definite relation to previous illumination.

9. Photosensory recovery after complete exhaustion occurs in the dark in about fifty minutes.

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Estudios sobre la espermatogénesis de los reptiles.

I. Sobre la espermatogénesis de los lagartos.

Los reptiles representan prácticamente un campo vírgen para el estudio del problema de los cromosomas sexuales. El present trabajo comprende la espermatogénesis de siete especies de lagartos pertenecientes a dos familias. El autor ha encontrado un cromosoma X del tipo "doble impar" (procedente de dos cromosomas espermatogoniales). Aparece como un cuerpo bipartido que pasa sin dividirse a uno de los polos del huso de la primera mitosis de maduración, dividiéndose ecuacionalmente en el segundo espermatocono.

Los espermatozoides son dimórficos, es decir, la mitad de ellos llevan y la otra mitad carecen del cromosoma sexual. Los cromosomas de la familia Iguanidae presentan una sorprendente separación en dos grupos de distinto tamaño. Existen invariablemente doce grandes cromosomas en forma de V y un número de corpúsculos redondos; cada especie posee su número característico. El autor no ha encontrado "doble reducción de los cromosomas.

Translation by José F. Nonidez
Cornell Medical College, New York

STUDIES IN REPTILIAN SPERMATOGENESIS

I. THE SPERMATOGENESIS OF LIZARDS¹

THEOPHILUS S. PAINTER

SIX TEXT FIGURES AND FOUR PLATES (FORTY-EIGHT FIGURES)

INTRODUCTION

Cytologists are well agreed that our knowledge of the chromosomes, especially of the sex-chromosomes, of vertebrate animals is in a very conflicting and unsatisfactory state. A critical review of the literature will show that, while the spermatogenesis of most of the common vertebrates has been worked upon, with a view of determining if sex-chromosomes were present, much of the work has been fragmentary in character, and various criteria have been used for identifying the sex-chromosomes (such as nucleoli in spireme stages, dimorphism of sperm, or the early movement of one chromosome to one pole in a maturation division). Too frequently conclusions have been based upon a few dividing first spermatocyte cells, without checking up such observations on cells of the following division or without a study of the female chromosome complex. In only a few vertebrates is the somatic or diploid number of chromosomes definitely known. Even in forms where the spermatogenesis has been most carefully and completely worked out, as in the pig or the opossum, there is a wide divergence of opinion as to the conditions existing. Thus Wodsdalek ('13) gives 18 as the diploid number of chromosomes for the male pig, while Hance ('17), using an improved technique, shows that there are 40 chromosomes both in the germinal and the somatic cells of this animal. Jordan ('12) gives 9 as the haploid number of chromosomes for the male opossum, while Hartman ('19), working upon ova, found 12

¹ Contribution no. 149 from the Department of Zoology, University of Texas, Austin, Texas.

to be the reduced chromosome number of the female. These two examples are typical of the confusion found at present in this field.

The difficulties which have confronted workers in vertebrate spermatogenesis are well known. The vertebrate germ cells have been, in the past, the most difficult of all tissues to fix properly for chromosome studies. The number of chromosomes in every case so far reported on is relatively large, and there is a very pronounced tendency for the chromosomes to fuse together in the spindles so that accurate counting usually has been impossible. Recently, however, through the investigations of Allen ('16, '19) and Hance ('17), some real advances in technique have been made, so that, with care, much of the difficulty experienced by earlier writers may be overcome. With the reinvestigation of the common vertebrates with these new methods, we may confidently expect that the present confusion will be cleared up to a large extent.

The reptiles are the only large class of vertebrates that have not been extensively studied by cytologists with a view of determining the condition of the sex-chromosomes. With the exception of a brief note by Jordan ('14) describing the results of his work on the spermatogenesis of the turtles, *Chrysemys marginata* and *Cistudo carolina*,² the reptiles present a virgin field for the investigator.

The following paper presents the results of an extensive study of the spermatogenesis of a number of lizards made during the past two breeding seasons, and, in addition, a study of dividing embryonic cells, made in order to check up certain conclusions regarding the sex-chromosomes.³ In making this study the author had two main objects in view: First, as we knew nothing about the sex-chromosomes of lizards, it seemed desirable to fill out this chapter of our knowledge. The second consideration was

² A complete account of this work has never been published. Professor Jordan, however, very kindly sent me his manuscript together with the figures, and from time to time I shall refer to the conditions found by him in the turtle.

³ The main facts concerning the spermatogenesis of *Anolis carolinensis* were presented before the American Society of Zoologists, December 28, 1919. See *Anat. Rec.*, vol. 17, for abstract of paper.

to determine what light, if any, a study of reptilian spermatogenesis would throw on the peculiar conditions found in the spermatogenesis of birds, as reported by Guyer in various works, since the reptiles and birds are closely related phylogenetically.

As the following study will show, certain species of lizards have been found in which the peculiar form of the chromosomes, as well as their small numbers, has allowed an accurate study of the spermatogenesis to be made; in fact, it has been found possible to follow most of the chromosomes from the last spermatogonial division through maturation into the immature spermatids. It must be confessed, however, that no facts have been discovered which throw any light on the unusual conditions reported for birds by Guyer ('16). The fact is, that during maturation the chromosomes of lizards behave in quite an orthodox manner, and differ in no essential respect from the behavior of the chromosomes of such a classic insect as *Lygaeus* during the corresponding period.

MATERIALS AND METHODS

Lizards embracing two separate families (Iguanidae and Teiidae) were studied. In the case of the Iguanidae, six species including five different genera are reported on. The family Teiidae is represented by only one species, in the vicinity of Austin, *Cnemidophorus gularis*.

In one respect the lizards offer very favorable vertebrate material for cytological studies. They have a very definite and short breeding season and in the males of many species the approach of this period is heralded by the appearance of the bright colors (blues and greens usually) which mark this sex during the mating season. This fact makes it easy to obtain and preserve the testes when division stages are most numerous.⁴

⁴ Within a radius of ten miles of Austin, one finds forms characteristic of the high-plateau areas as well as those forms which frequent the valleys and moist areas. Capturing members of some of the species alive is no easy task, as they run with what appears to be lightning-like speed. The author is indebted to two students of the Zoological Department, Mr. Kenneth Cuyler and Mr. Deluz

While a number of fixing fluids were tried, the author has obtained his best fixation by using cold Flemming's solution with urea, after the method of Hance, and Allen's modification of Bouin's fluid. The methods of hardening and dehydration as described by Hance ('17) and by Allen ('19) were followed. Of these two methods the modified Bouin's fluid on the whole gave the better results. However, my experience has been that, for somatic or spermatogonial divisions, the cold Flemming method gives a better separation of the chromosomes.

In all cases the spinal cord of the lizard was cut just behind the skull. The abdomen was then opened, the testes removed, split open, and immediately put into the preserving fluid, where the tubules were teased apart. This insured rapid and complete fixation.

The lacertelian testis is made up of convoluted tubules, much the same as have been found in all higher vertebrates. In general the various cell generations may be found as follows: spermatogonia lie on the periphery of the tubule, just within this outer circle one finds the first spermatocytes in their various stages, and the second spermatocytes and spermatids surround the lumen of the tubule. Exceptions to this arrangement are numerous, especially in the testes of mature males, but one has no difficulty in distinguishing cells of the various generations, since the size of the cells and the shape of the chromosomes are markedly different in these several stages. Supporting cells are commonly seen in the walls of the tubules, but I have failed to find anything which could be identified as true Sertoli cells.

In all the lizards studied, and this is especially true of the Iguanidae, the chromosome complex is made up of two sets of bodies strikingly different in size. In figure 1 a dividing spermatogonial cell is shown. It will be noted that there is an outer circle of large V-shaped chromosomes, while within the center of the spindle is filled with a number of dot-like chromosomes.

Hamblett, for catching many of the males upon which this study was made. Through their knowledge of the habits of the commoner species, together with their agility, they were able to run down and catch some forms which otherwise I should not have been able to obtain. Due acknowledgment is made to these gentlemen in this place.

The same general arrangement is retained through the maturation divisions and is typical for all Iguanidae studied. For convenience in the description and the discussion, the term 'macro-chromosome' has been adopted to designate the large V-shaped bodies, and the term 'micro-chromosome' has been applied to the small dot-like bodies. It should be emphasized, however, that the use of these terms is for convenience in descriptions, and that no physiological or functional difference between the macro- and micro-chromosomes is implied. Indeed, in one species studied, there is really no very sharp line to be drawn between the two sets of bodies, and in every case both types behave in the same way, except for the sex-chromosomes.

The Iguanidae proved to be the most favorable family for study, and among the species examined *Anolis carolinensis* and *Sceloporus spinosus* gave the best preparations.

The local fauna around Austin is rich in genera and species for several families of lizards. Material has been preserved and studied for the following species, common local names are indicated:

<i>Anolis carolinensis</i> ,	'American chameleon'
<i>Holbrookia texana</i> ,	'Zebra-tail lizard'
<i>Crotaphytus collaris</i> ,	'Mountain boomer'
<i>Uta ornata</i> ,	'Rock lizard'
<i>Sceloporus spinosus</i> ,	'Tree lizard'
<i>Sceloporus undulatus consobrinus</i> ,	Rare
<i>Cneminophorus gularis</i> ,	'Race runner'
<i>Phrynosoma cornutum</i> ,	'Horned toad'
<i>Gerrhonotus liocephalus</i> ,	Rare

In the following pages the spermatogenesis of the first seven species will be given. Much time has been spent preserving and studying the testes of the 'horned toad,' but no first-class preparations were obtained. The same may be said also of *Gerrhonotus*, but only one male of this species was examined, and he was too mature to be favorable for study.

FAMILY IGUANIDAE

Spermatogenesis of Anolis carolinensis

Spermatogonial divisions. All of my material was from fully mature testes, so that probably all dividing spermatogonial cells observed would have formed primary spermatocytes. Seen from the equatorial plate view dividing spermatogonia (figs. 1 to 3) show an outer circle of large V-shaped chromosomes (macro-chromosomes) surrounding the dot-like micro-chromosomes which lie scattered about the center of the spindle.

The macro-chromosomes lie well apart, with very little overlapping and no fusion of the elements. The V's show, typically, no trace of an achromatic bridge between the two arms, although, in figure 1, the 'b' chromosomes seem to indicate this. Repeated counts of spermatogonial division stages, such as are shown in figure 1 and 3, indicate that there are twelve macro-chromosomes in every spindle. (Careful drawings of over thirty cells were made, and each gave this number; in addition, innumerable other counts have been made, always with the same result.) While the shape of the macro-chromosomes makes it somewhat difficult to pair up synaptic mates, one can usually distinguish three pairs, on the basis of size. Two chromosomes, labeled 'a' (figs. 1 and 2), are larger than the rest (best seen in fig. 2). The other macro-chromosomes, labeled 'b,' are decidedly smaller than the rest, while a third pair, labeled 'c,' are slightly larger than 'b,' but smaller than the remaining six chromosomes, which are much the same size and shape.

The micro-chromosomes are small dot-like or, in some views, very short rod-like elements, which lie well separated in the center of spindles. There is some variation in the size of various dots (fig. 1) and, in a general way, one can mate up these chromosomes. Making accurate counts of the number of micro-chromosomes is made difficult because one or more frequently lie close to a macro-chromosome, and there is always a chance that one or two elements will be hidden and overlooked. The full number seems to be 22 (fig. 1), although in figures 2 and 3 we find 21 and 20, respectively. Occasionally these small

bodies are connected up, more or less, by strands of lightly staining material (fig. 3), which increases the difficulty of counting.

The spermatogonial division is completed in the usual way, both the macro- and micro-chromosomes dividing. There is no lagging behind of any elements, so far as I have observed.

First maturation division. No especial effort has been made to follow in detail the changes which the chromosomes undergo from the telophase of the spermatogonial division up to the prophase of the first maturation division, although the material is such that this probably could be done with comparative ease. Only a general outline of the sequence of stages is given here.

Following the last spermatogonial division, the young spermatocyte enters the diffuse stage (fig. 4), at which time the nucleus is characterized by the presence of two large deeply staining nucleoli and scattered chromatin knots lying on the linin net-work. No plasmosomes have been found at this time. Apparently the formation of the leptotene threads comes about, first, by an increase in the size of the chromatin knots and a decrease in the size of the nucleoli, and following this, the chromatin knots expand into the filament-like leptotene threads. The nucleoli still persist as deeply staining points in the general mass of chromatin threads, although they are small. No attempt was made to follow the course of events during synapsis, but it has been noted that no contraction stages occur in *Anolis* or any of the other lizards studied, nor is there any marked polarization of the nucleus following. The diplotene nucleus (fig. 5) is characterized by the thick spireme threads and the presence of two deeply staining elements (marked X), which appear to be more or less elongated. As the diplotene threads contract to form the tetrads of the first maturation division, these deeply staining elements form a conspicuous bipartite body, which has a compact form and smooth outline, while the rest of the chromatin elements are still much elongated. Figure 6 shows the condition of such a cell.

During this period just described, no trace of the small micro-chromosomes has been found. They apparently form spireme threads like the macro-chromosomes, and, indeed, in some cells

one finds short strands of chromatin (fig. 5, just above the deeply staining X-elements) which may represent micro-chromosomes.

The tetrads, which condense into the prophase chromosomes, are for the most part ring-shaped, at least this is true for three of them. These rings subsequently divide so that a V-shaped element goes to each pole, but I have not determined which plane the line of splitting cuts through. Among the rings the conspicuous bipartite X-body can readily be seen.

When the first maturation division spindle is formed, one sees in equatorial plate view (figs. 7 and 8) six large macro-chromosomes and eleven micro-chromosomes. Among the macro-chromosomes the small 'b' chromosome can always be identified, the 'c' chromosome is frequently seen, but it not so easy to mark the 'a' chromosome in polar view. The presence of six macro-chromosomes in the first maturation division indicates that the twelve spermatogonial chromosomes have united, and the presence of eleven micro-chromosomes (there are twenty-two in the spermatogonia) indicates that the same thing holds true for these smaller bodies.

Side views of the first maturation division show several points of considerable interest (figs. 9 to 14). First of all, one can nearly always identify four chromosomes in such views. The large 'a,' the small 'b,' the intermediate 'c' chromosomes show up in all such views, and in addition a bipartite chromosome is observed (marked X) which has spindle fiber attachments at one pole only of the cell. The micro-chromosomes (figs. 10 to 13) show up as small dumb-bell-shaped bodies, which are doubtless small tetrads.

As the first maturation division goes forward, one finds here and there cells in which the bipartite chromosome (marked X) is passing undivided to one pole. Figures 9 to 14 show six such cells. A close study of the chromosomes has shown that it is the same chromosome which shows this movement in the different cells, and, further, that this bipartite element is not half of one of the tetrads which has divided early, the other half remaining in the spindle. This bipartite element is believed to be the same

that has been seen in the earlier spireme stages, and so it has been marked *X* in all cases.

In this place it should be emphasized that this early migration of the *X*-chromosome is not seen in all side views of the spindles. One may examine several dozen cells without finding the *X*-element lying far out of the division plane, although, after a little experience, it can usually be recognized. But again one may find a patch of dividing cells in which the *X*-element will show up conspicuously, as in figures 9 to 14. This probably means either that the *X*-element goes to one pole a little in advance of the other chromosomes or that typically it moves at the same time the other elements divide, but occasionally may go early to one pole. In either case the infrequency of the phenomenon would be explained.

Aside from the fact that the *X*-element does not divide, the first maturation division proceeds normally. Late anaphases show five macro-chromosomes at one pole and six at the other (figs. 15 to 17), the extra chromosome being the bipartite *X*-chromosome.⁵ Thus the study of anaphase stages bears out the observations that the bipartite element passes to one pole of the cell undivided. Hence half of the second spermatocytes will carry, and half will lack the *X*-element.

Second maturation division. Following the first spermatocyte division, the chromosomes of the young second spermatocyte enter into a spireme condition. No conspicuous nucleoli have been observed in such resting nuclei. When the chromosomes of the second division condense out of this resting nucleus, they are generally already split in the plane in which they are destined to divide. This precocious splitting (fig. 18) makes it somewhat difficult to make accurate counts in the equatorial plate view, because one does not know whether a given chromatin mass is one chromosome already split or two chromosomes lying side by side. Figures 19 to 21 show three cells in which there are five macro-chromosomes. Figures 19 and 20 are

⁵ In figures 17 and 21 the cell was viewed from one pole, so that in order to show all the chromosomes, one pole was drawn, and then the cell shifted with the mechanical stage until the other pole was clear of the first.

equatorial plate views, while figure 21 is an anaphase stage. Figures 22 to 24 show three cells in which six macro-chromosome elements are present. In equatorial view (figs. 22 and 23) the additional X-element appears either as quadripartite (fig. 22) or as a large bivalent chromosome. An anaphase stage (fig. 24) shows six macro-chromosomes at each pole of the cell. This would indicate that the X-element divides equatorially at this time.

It will be noted that, in all the equatorial views of several division stages, a number of micro-chromosomes are seen. In figure 19 there are clearly ten elements and a blurred mass, which is part of the faintly staining network found in all spindles. In figure 20 there are eleven micro-chromosomes, one of which is larger than the rest. In figure 21 it is clear that eleven micro-chromosomes are going to each spermatid. Figure 23 again shows eleven micro-chromosomes.

From the foregoing description it will be seen that, in *Anolis carolinensis*, we have a bipartite chromosome which goes undivided to one pole of the cell in the first maturation division. Its behavior is typical of a so-called sex-chromosome or X-chromosome, and has been so regarded by the author. Later in this paper further evidence for this conclusion will be presented.

It is interesting to note, further, that the chromosomes of *Anolis* behave in a way typical for insects and other invertebrates. The tetrads are formed and divide in the typical invertebrate manner, and the second spermatocyte division does not show any fusion, or so-called 'double reduction,' such as has been described for birds and some mammals.

The transformation of the spermatids into mature sperm has been followed out, but no description of it will be given here. Giant sperm are occasionally found in *Anolis* and so-called 'syncytial masses' are common. These will be treated in a later section of this paper.

Spermatogenesis of Sceloporus spinosus

This common 'tree lizard' has proved to be a very satisfactory form upon which to work, because the germ cells are large and preserve well with either cold Flemming or modified Bouin; there are relatively few micro-chromosomes to be found in the spindles and they are large, and the X-chromosome element is seen passing undivided to one pole in the first maturation division more often than in any other lizard studied. As a glance at the figures of plate 3 will show, the behavior of the chromosomes in *Sceloporus spinosus* during maturation is closely similar to what is found in *Anolis carolinensis*.

Spermatogonia. Dividing spermatogonia (figs. 25 to 27) show a condition strikingly similar to that of *Anolis*. In equatorial plate view there are twelve macro-chromosomes, forming a general circle, and ten micro-chromosomes lying scattered over the center of the spindle.

A close study of the macro-chromosomes shows one pair longer than the rest, labeled 'a,' a small pair, labeled 'b,' markedly smaller than the other V's, and in figure 25 a third pair, 'c,' is seen, somewhat larger than 'b.' (Fig. 25 is a cell preserved in cold Flemming, while figs. 26 and 27 are taken from cells preserved in Allen's modification of Bouin.) The six remaining chromosomes are all about the same size. A comparison of figures 25 and 26 with figures 1 to 3, taken from *Anolis*, shows that, as regards macro-chromosomes, the two forms are similar in number and general size relations. The 'b' chromosome pair of *S. spinosus* is relatively much smaller than the corresponding 'b' chromosomes of *Anolis*.

The micro-chromosomes of *S. spinosus* are ten in number and are comparatively large in size. In addition to the rounded balls one also finds somewhat elongated blunt rods, as shown in the figures.

First maturation division. No detailed study was made of the various phenomena exhibited by the nuclear elements up to the prophase of the first division. A casual examination of

the preparations showed that *S. spinosus* differs in no marked respect from *Anolis*.

In equatorial plate views of the first maturation spindle there are six macro-chromosomes and, typically, five micro-chromosomes. This shows that there has been a pairing of all the spermatogonial elements. Considering first the macro-chromosomes, we have little difficulty in identifying the 'a,' 'b,' and 'c' chromosomes, and in addition a bipartite chromosome labeled X. Generally, all the macro-chromosomes, except the X and the 'b' chromosomes, show their tetrad character (figs. 28 and 30). The micro-chromosomes are much larger than similar bodies in *Anolis*; in fact, in *S. spinosus* there is less difference in size between the largest micro-chromosome and the 'b' chromosome than between the 'b' element and any of the macro-chromosomes, excepting 'c.' Frequently one of the micro-chromosomes lies so that both halves of it may be seen (figs. 28 and 30).

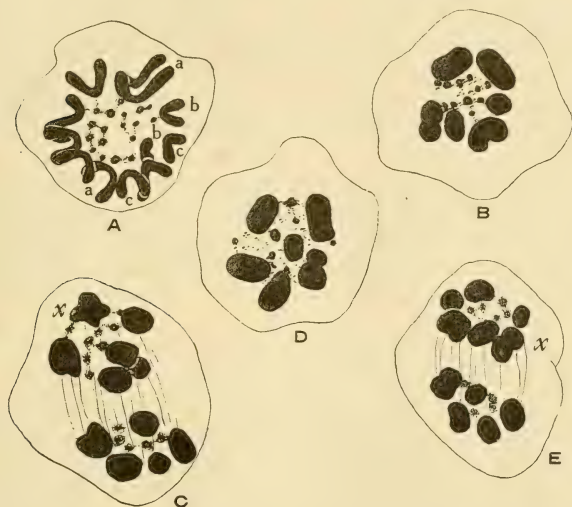
In side views of the first maturation spindle one sees in perhaps a third of all the cases a bipartite chromosome (marked X) passing undivided to one pole of the cell. The size and shape of this X-element varies somewhat in different cells, but its bipartite character is strongly marked in every case (figs. 31 to 33). In other respects the first division goes forward normally. In figure 33 the 'b' chromosome and several of the micro-chromosomes have divided.

On the basis of the first division, one should expect that the secondary spermatocytes would be of two kinds, half with, and half without the X-chromosome.

Second maturation division. The precocious splitting of the chromosomes as they come out of the resting nuclear stage makes a study of second spermatocyte divisions difficult. In favorable cells, however, one may clearly make out either five or six macro-chromosomes. Figure 34 shows a cell with five, and figures 35 and 36 show cells with six large chromosomes. The X-element is marked in figure 36.

Spermatogenesis of Sceloporus undulatus var. consobrinus

The spermatogenesis of this species was carried out to the extent of determining the chromosome complex and the behavior of the sex-chromosome. This small lizard is rather rare around Austin. My material was taken from two males.



Text figure 1 *Sceloporus undulatus var. consobrinus*. A. Spermatogonial plate. B and D. Equatorial plates of the first maturation division. C and E. Late anaphase stages of the first division.

In text figure 1, A to E, the essential features of the spermatogenesis are shown. Dividing spermatogonia (A) show the typical outer circle of twelve macro-chromosomes, and within this the micro-chromosomes which number about eighteen. A careful examination of the macro-chromosomes shows that we have here essentially the size relations found in *Anolis* and *Sceloporus spinosus*. The 'a,' 'b,' and 'c,' chromosomes are labeled. (In text fig. 1, A the size difference between 'b' and 'c' is not well shown. It is more striking in the next division.)

The first spermatocyte cells show (text fig. 1, B and D) six large macro-chromosomes and from eight to eleven micro-chromosomes. Here, again, we can easily identify the 'b' and 'c' chromosomes, but the 'a' element is not so easily distinguished. A bipartite chromosome is also seen in such a view. No side views of the spindles are shown, but the two late anaphase stages represented in text figure 1, C and E, show what takes place in this division; the lower pole of each cell shows five macro-chromosomes, while the upper pole shows six large bodies. The extra element (marked X) appears sometimes as quadripartite (text fig. 1, C), sometimes as a bivalent chromosome (text fig. 1, E).

No study of dividing second spermatocytes was made. Judging, however, from the anaphase stages of the first division, half of the secondary spermatocytes should carry five and half should carry six chromosomes, the extra element being the X-chromosomes. In this respect, *S. undulatus consobrinus* is like *Anolis* and *S. spinosus*.

Spermatogenesis of Holbrookia texana

This 'zebra-tail' lizard has not proved a very satisfactory form for study, because the testes, in my experience, are difficult to preserve well and the sex-chromosome rarely shows up in side views of the first maturation spindle; that is, it rarely passes to one pole of the cell before the autosomes divide. Spermatogonial division stages are rare in my preparations, but favorable plates (fig. 37) show an outer circle of V-shaped macro-chromosomes, while within the micro-chromosomes are found. A close inspection of figure 37 will show the presence of three pairs of macro-chromosomes which are distinctive because of their size. These are labeled 'a,' 'b,' and 'c,' just as was done for the other lizards studied. The micro-chromosomes do not show up well in figure 37.

In equatorial plate view the first maturation spindles show six macro-chromosomes and about twelve or thirteen micro-chromosomes (figs. 38 and 39). There is a marked tendency

for the tetrads to separate in the plane in which they will be divided later. One of the macro-chromosomes appears as bivalent.

Side views of the first maturation division very rarely show the condition reproduced in figure 40. The presence of a bipartite chromosome at one pole strongly suggests a condition similar to that found in the other lizards studied, but I am unable to state definitely whether or not we see the true X-element in figure 40. Late anaphase stages show the conditions reproduced in figures 41 to 43. In figure 41 we see a pair of chromosomes projecting out from the upper pole of the cell. Figures 42 and 43 show six large chromosomes at the upper pole and five chromosomes at the lower pole, the additional element at the upper pole being marked X. (Fig. 43 is taken from poorly preserved material.) This condition would indicate that an X-chromosome is present in *Holbrookia* and that it goes undivided to one pole in the first maturation division just as was found in the other lizards described.

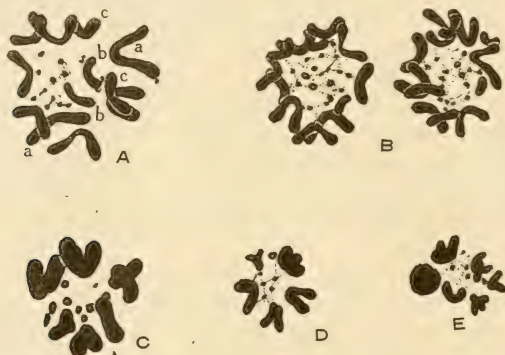
The evidence from the second spermatocyte division bears out the conclusions drawn from the first division. Figure 44 shows a side view of a spindle, in which one sees the precocious splitting of the chromosomes. Figure 45 is an equatorial plate view showing five macro-chromosomes, and figure 47 is a late anaphase showing five macro-chromosomes going to each spermatid. (In figs. 47 and 48 the cells were viewed from one end, so in order to show both poles the cells were shifted with the mechanical stage after one pole was drawn.) Figure 46 shows a cell with six macro-chromosomes, and in figure 48 we have a late anaphase in which six macro-chromosomes are seen distinctly going into each spermatid.

From this study it is clear that the spermatogenesis of *Holbrookia texana* differs in no essential respect from what was found for *Anolis* and *Sceloporus*.

Spermatogenesis of Uta ornata

The study of the spermatogenesis of this form has been carried out sufficiently to give the chromosome complex and the behavior of the sex-chromosome.

Spermatogonial divisions (text fig. 2, A and B) show the presence of twelve macro-chromosomes and from fifteen to eighteen micro-chromosomes. Among the macro-chromosomes we distinguish a large pair, labeled 'a,' a small pair, 'b,' and the 'c'



Text figure 2 *Uta ornata*. A and B. Dividing spermatogonia. C. Equatorial plate view of a dividing first spermatocyte. D and E. Equatorial plate view of dividing second spermatocytes.

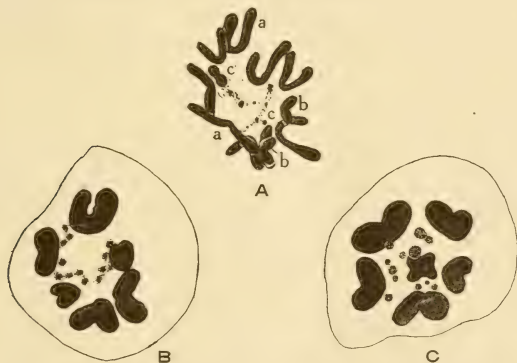
pair as found in the other lizards of the family Iguanidae (text fig. 2, A). The 'b' chromosomes are relatively large in this species.

The first maturation spindles show six macro-chromosomes and about eight or nine micro-chromosomes (text fig. 2, C). In side views of the spindles I have not observed the early migration of an X-element to one pole. However, the secondary spermatocytes contain five (text fig. 2, D) and six (text fig. 2, E) macro-chromosomes, the extra element being large and represents, no doubt, the X-chromosome. Hence it is very probable that a sex-chromosome is present in *Uta ornata* and that it behaves like this element does in *Anolis*.

Spermatogenesis of Crotaphytus collaris

The study of this lizard was undertaken to determine the chromosome complex, and no attempt was made to trace out the history of the chromosome elements during maturation.

There are twelve macro-chromosomes (text fig. 3, A) in the spermatogonial divisions and a number of micro-chromosomes. Among the macro-chromosomes one recognizes the 'a,' 'b,' and 'c' elements described for other Iguanidae.



Text figure 3 *Crotaphytus collaris*. A. Dividing spermatogonia. B and C. Equatorial plate of the first maturation division.

The first spermatocytes contain six large macro-chromosomes and around twelve micro-chromosomes (fig. 3, B and C).

FAMILY TEIIDAE

The local representative of this family is the common 'race-runner,' *Cnemidophorus gularis*, which is perhaps the commonest of all the species of lizards found near Austin. The germ cells of this form, however, have proved very difficult to fix properly, and although cold Flemming and modified Bouin's fluid have been used repeatedly, I have never obtained first-class preparations which would allow me to follow the complete history of the chromosomes through maturation. Since, however, *Cnemido-*

phorus represents an entirely different family from the lizards so far described, a fragmentary account of the spermatogenesis will be included here.

Spermatogonial divisions have been studied, but the number of chromosomes involved and the poor fixation made it impossible to determine even the approximate number of these elements.

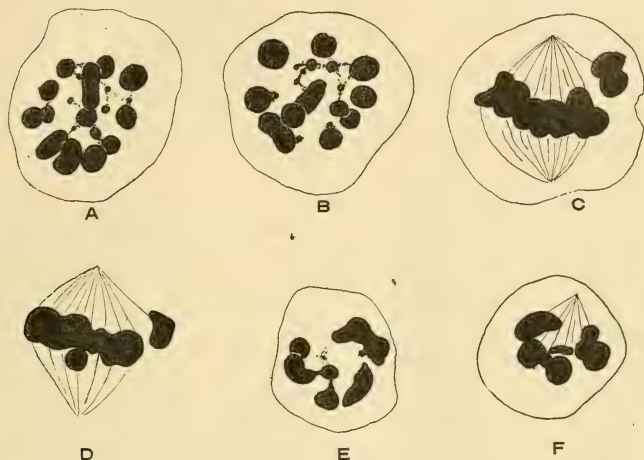
The early history of the first spermatocytes was followed in some detail, and was found to differ in no way from what had been observed in *Anolis carolinensis*. Two large nucleoli are seen in the 'diffuse' nucleus; these diminish in size as the leptotene threads appear. In early prophase stages the chromosomes generally appear as rounded masses, and one cannot see the formation of tetrads, as could be done in the family Iguanidae. This is due to the faulty fixation of my material.

In the primary spermatocyte division stages the chromosomes appear as rounded masses of varying size (text fig. 4, A and B) about twenty in number. Of these, thirteen are large, while seven are much smaller. It will be seen from the figures, however, that the large and small chromosomes intergrade in size and we do not have sharp division into macro- and micro-chromosomes such as was found in the family Iguanidae.

In side views of the first spermatocyte spindles one occasionally finds cells such as are shown in text figure 4, C and D. A bipartite body lies to one side of the spindle, well advanced (text fig. 4, C) toward one pole. This element is very suggestive of the X-element found among the other lizards studied, but I was unable to make counts of anaphase stages, so that this point could not be verified.

In the second spermatocyte division (text fig. 4, E and F) the chromosomes have fused together, so that accurate counting is out of the question. This fusion of elements is, perhaps, similar to the 'double reduction' reported in the second spermatocytes of birds and of some mammals. *Cnemidophorus* is the only lizard, out of seven species studied, in which I obtained such fusion. The chromatin masses vary in number from five up to ten or more, the variation being due to more or less general fusion of the nineteen or more chromosomes which were handed

on from the first spermatocyte. This fusion is the result of poor fixation, which made the spermatogonial division unworkable.



Text figure 4 *Cnemidophorus gularis*. A and B. Equatorial plate views of dividing first spermatocytes. C and D. Side views of the first maturation spindle. E and F. Views of the second spermatocyte cells showing fusion of chromosomes.

THE FEMALE CHROMOSOME COMPLEX

A study was made of the ovarian tissue of both *Anolis carolinensis* and *Sceloporus spinosus*, with a view of determining the chromosome complex of the females of these two species. Very small young females were used, the ovaries being preserved and sectioned. As was to be expected, no oogonial divisions were found, but scattered here and there among the nurse cells were dividing cells. A great number of these were carefully studied, but most of the equatorial plates were too crowded to allow more than approximate counts. Here and there, especially in cells which had been cut in two by the sectioning razor, the chromosomes lay fairly well apart, so that counts could be made.

In such cases I found typically fourteen macro-chromosomes, though the counts varied between thirteen and fifteen. In only two or three cases did I find dividing cells in which I was confident that there was no error in my count, and these gave fourteen macro-chromosomes as the female complex.

The evidence for the conclusion that in females of both *Anolis* and *Sceloporus* there were fourteen macro-chromosomes was so meager that a study of the dividing somatic cells of embryos was undertaken in order to get further light upon this point.

At the outset the author was aware that chromosome counts made on dividing somatic cells may not be accepted unhesitatingly as proof of the number of chromosomes which will be found in the germ cells of the individual. A number of investigators have shown both for vertebrates and invertebrates⁶ that the number of somatic chromosomes may vary from that which one would expect from a study of the germ cells. More recent work has indicated, however, that this variation in chromosome number is either due to a fragmentation of one of more of the chromosomes (Hance, '17) or that we have multiples of the haploid number (Holt, '17) due to a longitudinal splitting of the chromosomes. Hance's work is of especial interest, in connection with lizard embryos, since from his study of dividing somatic cells of the pig he was able to show that variations from the diploid number (forty) were due to the fragmentation or breaking of some of the normal chromosomes, and not due to the addition of new chromatin elements.

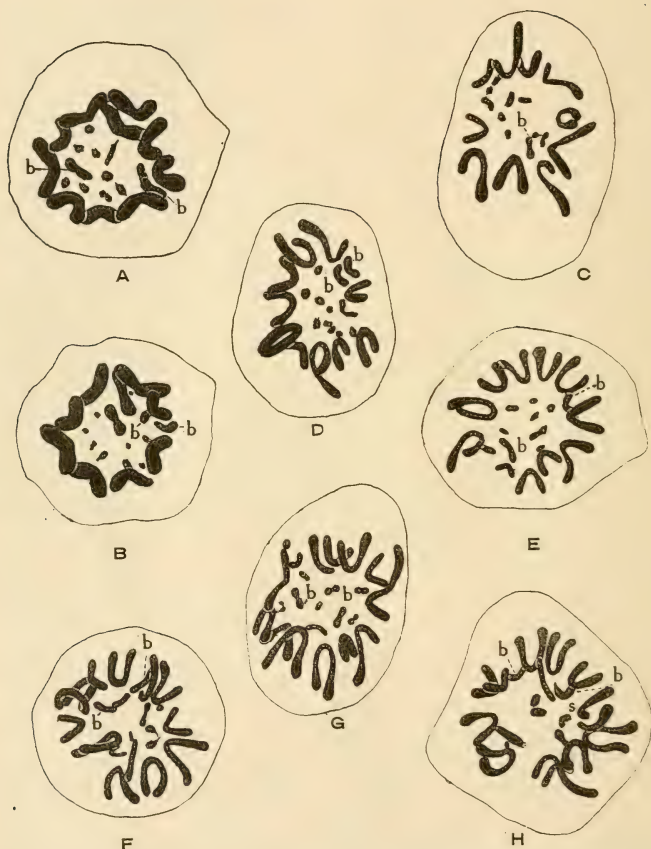
In *Sceloporus spinosus*, the form upon which the following work was carried out, the large macro-chromosomes have fairly characteristic shapes (V's of various sorts) and there are relatively few of them. On the basis of the study of spermatogenesis, it was clear that the X-element found in the first maturation division was derived from two medium-sized spermatogonial macro-chromosomes. It was believed that two extra V's could be detected, if they were present in the females.

⁶ An excellent review of the literature dealing with this point is given by Hoy ('16).

(If the conclusions drawn from a study of spermatogenesis are correct, then the female of this species should have fourteen macro-chromosomes, that is, two extra chromosomes, which are equal to an additional X-chromosome. See p. 307 for further discussion of this point.)

Before examining the somatic divisions of embryos, the chromosome complex of the male germ cells should be carefully examined. In the adjacent text figure 5, A and B (also plate 3), we have drawings of two spermatogonial divisions. It will be noted that there are ten large conspicuous chromosomes which have a V shape, two small V's labeled 'b,' and ten small elements, the micro-chromosomes. It is not always easy to distinguish the small 'b' chromosomes from the largest micro-chromosomes, because occasionally, and this is especially true of somatic divisions, the larger micro-chromosomes also have a V shape. The ten large macro-chromosomes, however, are conspicuous and easily identified, and since the X-element comes from a pair of these, we may disregard the 'b' chromosomes and the micro-chromosomes in the following description. If the X-element has been correctly identified in the spermatogenesis, then the female should show twelve large V-chromosomes and the males ten.

The four embryos, from which the cells shown in text figure 5, C to H, were taken, were all obtained from one female, preserved in the same dish of cold Flemming, carried through dehydration in the same vial, embedded in the same paraffin block side by side, and stained on the same slide. In studying embryos, one individual at a time was followed, camera drawings made of the chromosome elements, and afterwards these were compared and checked up. The number of large V-shaped chromosomes was constant for a given embryo. Occasionally one of the V-elements was apparently broken in two, but as the two halves were adjacent, it caused no confusion (text fig. 5, H). I did find, however, some variation in the shape, size, and number of the micro-chromosomes in the same individual. In one cell, for example, the normal number (ten) of micro-chromosomes would be seen, while in an adjoining section a cell would be



Text figure 5 *Sceloporus spinosus*. A and B. Spermatogonial chromosomes. C and E. Somatic chromosomes of first embryo. D. Somatic chromosomes of the second embryo. F and G. Somatic chromosomes of a third embryo. H. Somatic chromosomes of a fourth embryo.

found where, apparently, several of these micro-chromosomes had joined to form one long thin rod. In no case was it difficult to distinguish between such thin rods and the V-shaped macro-chromosomes.

Figures C and E show the characteristic condition of the somatic chromosomes for one embryo. In text figure 5, C, ten large V-shaped macro-chromosomes are seen, together with eleven smaller bodies. Of the latter one is a 'b' chromosome, its mate is not identified. In text figure 5, E, there are also ten large chromosomes, two 'b' chromosomes, and ten or eleven micro-chromosomes (depending on how we interpret the small bipartite bodies).

The somatic chromosomes for a second embryo are shown in text figure 5, D. Here there are ten macro-chromosomes, a pair of 'b' chromosomes, which show with unusual clearness, and ten or eleven micro-chromosomes.

The chromosome complex of a third embryo is shown in text figure 5, F and G. Here one finds at least twelve large V-shaped chromosomes. In the cell shown in text figure 5, F, the 'b' chromosomes may be identified, but instead of the dot-like micro-chromosomes, one sees some elongated rods representing probably several of these small bodies fused together. The cell shown in text figure 5, G, taken from the same embryo, shows very clearly the twelve large V's, the two 'b' chromosomes, and at least ten micro-chromosomes.

The chromosome complex of a fourth embryo is shown in text figure 5, H. Here again there are twelve large V-shaped chromosomes. Of these, one (marked 's') appears to have broken in two. The 'b' chromosomes are less certainly identified, but are probably as labeled. Elongated rods represent, no doubt, fused micro-chromosomes.

The figures given above are typical for what one finds in each embryo. There is never any difficulty in identifying the large V-shaped chromosomes, but, as was noted for *Anolis*, there is a tendency for the micro-chromosomes to fuse together in some cells. This is not constant for an individual, however, as a comparison of text figure 5, F and G, will demonstrate.

Many embryos, besides those figured, were studied, the results being consistent throughout. *Sceloporus spinosis* embryos are of two types, one carrying ten large V-shaped macro-chromosomes (and the smaller V-shaped 'b' chromosomes) and the other carrying twelve large V-shaped elements and the 'b' chromosomes. The former are destined to be males, the latter are females.

DISCUSSION

1. *Sex-chromosomes*

At the present time the point of most general interest in works dealing with vertebrate spermatogenesis is the question: How is sex determined, what is the condition of the sex-chromosomes? Ever since the sex-chromosomes were discovered in the insects, a number of investigators⁷ have worked upon nearly all of the common vertebrates with a view of determining this question. In some cases a sex-chromosome has been reported, in other cases the results were negative, but it may be said with fairness that in only a few instances has the evidence for either the presence or the absence of the X-chromosome been complete enough to be convincing to one critically disposed toward the sex-chromosome theory. In perhaps the majority of cases the evidence for the existence of an X-chromosome has been based on observations of usually first spermatocyte cells, in which typically a bipartite chromosome was seen lying well towards one pole of the spindle and going presumably undivided to it. Only rarely have such observations been extended to the second maturation and proper check counts made here. And yet in view of all we know about the irregular way (in point of time) in which vertebrate chromosomes divide, it would seem that we must accept with some reserve those works in which the presence or absence of the sex-chromosome was concluded from a study of the first maturation division alone. It has been a matter of common

⁷ No better review of this subject could be given than is contained in a recent paper by Ethel Brown Harvey ('20). To the papers listed by Mrs. Harvey one should add the recent work of Wodsedalek ('20), dealing with the spermatogenesis of cattle.

experience, noted by many observers and found in the lizards, that vertebrate chromosomes are variable in the time at which they divide; this may be early or late, each body acting more or less as an independent entity. The result is that while a chromosome lying outside of the equatorial plane may be a sex-chromosome, it may also be half of a tetrad which has divided, the other half remaining in the equatorial plane, or it may be simply some chromosome displaced mechanically, either by the preserving fluids and subsequent treatment or by the sectioning razor. When the number of chromosomes in the spindle is large, so that one cannot identify each one of them in side view, then he can never be sure which phenomena he is observing.

Because of the peculiar form of the chromosomes in the family Iguanidae, these lizards have been extremely favorable for chromosome study. The total number of chromosomes in this family is large, but, happily, the macro-chromosomes, to which the X-chromosome plainly belongs, are small in number (twelve in the spermatogonia and six in the first maturation division). In side views of practically every complete maturation spindle, all of the large elements can be readily seen and three or four of them identified by their size and shape. (This applies to the 'a,' 'b,' and 'c' chromosomes and the X-chromosome.) During the course of this study, the author has observed, at one time or another, every one of the chromosomes besides the accessory so displaced as to appear to be passing to one pole of the cell undivided. Repeatedly cells have been found in which one of the tetrads has divided early, with one half of it lying well towards one pole while the other half was still to be seen in the equatorial plane. (This was often true of the small 'b' chromosome.) Such observations emphasize the necessity of checking up conclusions made on the first maturation spindle, in order to be sure that a real sex-chromosome has been found.

There are, of course, five points which should be determined in order to be sure of this. They are: *a*) the diploid number of chromosomes for the male; *b*) the haploid number of chromosomes in the first division and their behavior; *c*) the number of chromosomes present in the second spermatocytes; *d*) the

number of chromosomes going to the spermatids; *e*) the diploid number of chromosomes for the female. Points *b*, *c*, and *d* must be known and points *a* and *e* are very desirable checks. For lizards it was possible to determine all five points for one species (*Sceloporus spinosus*) and the first four points for two others (*Anolis* and *Holbrookia*). In the remaining species of the family enough points were determined to be sure that they conformed to the family type. The results have been consistent throughout.

In all the lizards studied two conspicuous nucleoli were found in the early growth period of the first spermatocytes. These persisted through the various spireme stages as deeply staining bodies and entered the prophase of the division as a bipartite chromosome. Side views of the first division spindle frequently showed this bipartite body lying outside of the equatorial plate and well up or quite up to one pole. Careful study of the six large chromosomes of the spindle showed that it was the same body in every case which showed this movement (except for the rare displacement of an autosome) and that it was not half of some tetrad which had divided early. On the other hand, it must be emphasized that this early migration of the bipartite chromosome is not seen in all spindles, and in some species it was not seen at all. This fact led to a very careful study of late anaphase stages of the first division, in order to find out if the X-chromosome really passed undivided to one pole. In every case where chromosome counts could be made, one pole contained one more macro-chromosome than the other, the extra element being a bipartite body similar in size and shape to the X-chromosome (figs. 15, 16, 17, 42, 43, and text fig. 1, C and E). This showed that the phenomena was a constant one, that one pole of the first maturation spindle received one more large chromosome than the other.

A study of the number of chromosomes in the second spermatocytes would seem to offer a simple way of verifying first spermatocyte conclusions, but, unfortunately, this did not prove to be the case with the lizards studied. When the chromosomes enter the second maturation spindle, they are already, in most cases,

precociously split, so that in equatorial view it is difficult to make counts. One does not know whether one or two chromosomes are involved. The matter is further complicated by the fact that the second spermatocyte cells do not preserve well (due perhaps to poor penetration). Under favorable circumstances, however, either five or six macro-chromosomes were found. Anaphase stages of this division were much clearer for study (because of the chromosome shape) and showed that the spermatids received either five or six large chromosomes (figs. 19 to 24, 34 to 36, and 45 to 48).

Among all members of the family Iguanidae which were studied, there were twelve macro-chromosomes in the spermatogonia, and six of these large chromosomes in the first maturation spindle. This shows that the bipartite body which acts like a typical X-chromosome is derived from two spermatogonial chromosomes. Since the male is heterozygous as regards sex, the female must be homozygous and have the two X composition. And since the X-chromosome of the male comes from two spermatogonial chromosomes, we should expect to find that the females had two more large chromosomes than the males ($X =$ two spermatogonial chromosomes; $2 X =$ four spermatogonial chromosomes).

A study of the female chromosome complex was made for *Sceloporus spinosus*, and while the results with the ovarian tissue were not altogether satisfactory, they indicated that the female has fourteen large chromosomes (the male of this species has twelve). A study of *Sceloporus* embryos, however, furnished convincing evidence on this point. As a glance at text figure 5 will show, some embryos show constantly two more large V-shaped chromosomes than the others. The embryos with the two extra chromosomes would have become females without doubt.

With this evidence at hand, we cannot escape the conclusion that a true sex-chromosome has been found in the lizards. This X-chromosome is of the 'double-odd' or the 'double accessory' type; that is, X equals two spermatogonial chromosomes.

The double-odd chromosome has been reported for other vertebrates (man, pig) and for a number of invertebrates. Wilson ('09) has reported this condition for *Syromastes marginatus*; Morgan ('15) describes it for certain Phylloxerans; Wallace ('05) and Painter ('14) found it in spiders; Goldsmith ('19)

TABLE 1

FORM	NUMBER OF CHROMOSOMES WHICH FORM SEX-CHROMOSOMES	APPEARANCE OF SEX-CHROMOSOMES	BEHAVIOR OF SEX-CHROMOSOMES	AUTHOR
<i>Rana esculenta</i>	1	Bipartite	To one pole 1st	Levy, '15
<i>Rana pipiens</i>	1	Bipartite	To one pole 1st	Swingle, '17
<i>Necturus maculosa</i>	Unknown	Rod att. to Autosome XY?	To one pole 1st	King, '12
Turtle	?	Bipartite	To one pole 1st	Jordan, '14
Chicken	2	Comma-like	To one pole 1st	Guyer, '09, '16
Guinea-fowl	1	Comma-like	To one pole 1st	Guyer, '09
Dog	1	?	To one pole 1st	Malone, '18
Cat	1	?	To one pole 1st	Winiwarter and Sainmont, '09
Armadillo	1	Bipartite	To one pole 1st	Newman and Patterson, '10
Opossum	1	Bipartite	To one pole 1st	Jordan, '11
Guinea-pig	2	XY condition	1st reductional	Stevens, '11
Rabbit	2	XY condition	1st reductional	Bachhuber, '16
Rat, albino	1	Bipartite	To one pole 1st	Allen, '18
Mouse, house	?	?	To one pole 2nd	Yocum, '17
Horse	1	Bipartite	To one pole 1st	Wodsdalek, '16
Cattle	1	Bipartite	To one pole 1st	Wodsdalek, '20
Pig	2	Bipartite	To one pole 1st	Wodsdalek, '13
Man	2	Bipartite	To one pole 1st	Guyer, '10
Man	1	?	To one pole 1st	Winiwarter, '12
Man	?	Bipartite	To one pole 1st	Montgomery, '12

reports it for Cicindelidae; Wieman ('10) found it in certain beetles; Guyer ('10) describes it for man, and Wodsdalek ('13) for the pig.

A point of considerable interest is a comparison of the sex-chromosomes, as I have found them in lizards, with similar bodies reported for other vertebrates. In table 1, the following

points have been tabulated for all cases of vertebrates where the sex-chromosomes have been seen in maturation spindles: first, the number of spermatogonial chromosomes going to make up the sex-chromosome; second, the appearance of the X-chromosomes in the maturation spindles, and, third, the behavior of the X-chromosome.

A glance at column 3 of this table will show that the first maturation division has been reported as the reductional division in nineteen out of twenty cases (the exception is Yocum's work on the house mouse). In lizards, of course, the X-chromosome does not divide in the first maturation division; hence this is the reduction division, as far as the sex-chromosomes are concerned.

The second column shows, in the vast majority of cases where we are dealing with the X-condition this chromosome is bipartite in form. The exceptions are birds and cases where the X-Y condition prevails. Again, in lizards, we invariably find the bipartite condition.

In the first column it will be noted that most observers describe the X-element as coming from one spermatogonial chromosome, and regard the bilobed form of the first maturation division as a precocious splitting in preparation for the second maturation division. A few workers describe the X-element as coming from two spermatogonial chromosomes (fowl, man, Guyer; and pig, Wodsdalek), and this is unquestionably the condition found in all the lizards studied. In lizards of the family Iguanidae the origin of the X-element from two spermatogonial chromosomes could be determined with great certainty; there can be no doubt that there are twelve macro-chromosomes in spermatogonia and six in primary spermatocytes. One of these six is the sex-chromosome.

Why should we find such close agreement in the appearance and behavior of the sex-chromosomes in vertebrates, and yet find this striking difference in the origin of the X-element? In this connection, it may be pointed out that while the number of chromosomes to be dealt with in lizards is small (twelve), other vertebrates show a range from seventeen to

fifty-six, and in very few instances was any author quite certain of his count. An error of one chromosome is not improbable.

The author is of the opinion that when the common vertebrates are restudied with improved technique, it will be found that the bipartite X-element arises from two spermatogonial chromosomes, and that its bipartite form during the first maturation division is an expression of this bivalency, and not the precocious appearance of the plane where the second maturation spindle will separate these elements.

2. Morphology of chromosomes in lizards

A very striking and interesting feature of the morphology of the chromosomes among lizards is the sharp separation into two size groups, which I have termed, for convenience in description, macro- and micro-chromosomes. This size relation, most conspicuous among the Iguanidae, is constant throughout maturation and in somatic divisions, although, as I have noted several times in this paper, there is some tendency for several micro-chromosomes to become associated together to form long thin rods.

The macro-chromosomes appear in somatic and spermatogonial divisions typically as equal-armed V- or U-shaped bodies. These are really 'bent-rod' chromosomes, as defined by Robertson ('16, p. 221), since there is typically no achromatic bridge between the two halves. In the first maturation division these macro-chromosomes form tetrads (except the X-element) with usually a ring or a double-cross form. In the second division we find bent rods or open V-shaped bodies going to either pole.

The micro-chromosomes appear typically as rounded dots in spermatogonial and maturation divisions, but in somatic divisions they may be elongated, short rods, or rarely small V's being formed. These small bodies unite during synapsis, so that bilobed or bipartite masses are found in maturation. All micro-chromosomes divide in a regular fashion and, as far as I can determine, the spermatids all receive the same number of them. Furthermore, their number seems to be constant in different individuals. This has been most carefully studied in

Sceloporus, where the testes of four males have been examined (after several fixatives), and in addition the somatic divisions of both male and female embryos. In all favorable cells the number of micro-chromosomes seemed to be ten.⁸

What significance does this sharp separation into two chromosome groups have? Is this merely a striking incident in the spermatogenesis of lizards, or are the macro- and micro-chromosomes physiologically unlike? May the micro-chromosomes be, perhaps, degenerating chromosomes which have outlived their usefulness in this ancient group?

From the evidence at present at hand, it appears that this separation into large and small chromosomes is merely an interesting incident of lizard spermatogenesis. This conclusion is based on the following observations: First, in the Teiidae, the size demarcation is not so sharp, the two groups grade into each other. Further, the micro-chromosomes behave as true autosomes, fusing in synapsis and dividing in a regular fashion in maturation. There is no evidence to show that their number is variable within the species. It is very probable that in the macro- and micro-chromosomes we are dealing simply with larger and smaller aggregations of chromatin matter.⁹

3. Chromosomes and taxonomy

In the family Iguanidae, where the spermatogenesis of six species of lizards has been studied, we have, as far as the macro- or large chromosomes are concerned, a confirmation of the general position taken by McClung ('05, '08) after his extensive studies of orthopteran chromosomes, that there is a definite relation between taxonomy and chromosomes; that the degree of relationship may be recognized in the germ-cells as well as in external characters. Robertson ('16) has recently given extensive confirmation to this view.

⁸ It will be understood, however, that there is a considerable chance for error in making micro-chromosome counts, for in addition to a chance hiding under other chromosomes, they tend to disappear on long extraction of the stain.

⁹ In *Drosophila* several genes have been found to exist in the small dot-like chromosomes.

Among all the Iguanids studied, the number of macro-chromosomes (involving considerably over 90 per cent of the chromatin matter) is always the same, namely, twelve, and, what is more remarkable, these chromosomes show the same size relations. There are always, in the spermatogonia, the pair of large 'a,' the small 'b,' and the intermediate 'c' chromosomes (see figures as follows: *Anolis*, figs. 1, 2, 3; *Sceloporus spinosus*, figs. 25, 26, 27; *Sceloporus U. consobrinus*, text fig. 1, A; *Holbrookia texana*, fig. 37; *Uta ornata*, text fig. 2, A and B; *Crotophytus* text, fig. 3, A). The six remaining chromosomes are too much the same size and shape to allow one to pair up accurately synaptic mates. So similar are the macro-chromosomes, of these six species that, except for the micro-chromosomes, the cell plate of one form might be easily substituted for another without its being detected.

On the other hand, the micro-chromosomes, representing in the aggregate less than 10 per cent of the chromatin, do not show this constancy in size and number. The number varies from ten to twenty-two, and even in closely related species, as *Sceloporus spinosus* and *S. U. consobrinus*, we have ten and eighteen, respectively, in the germ cells. Why should the macro-chromosome be so constant in size and number and the micro-chromosomes so variable? Two possibilities suggest themselves. One is, that in lizards we are witnessing the formation of a small number of large chromosomes through the fusion of the small bodies with the macro-chromosomes, and that this process has gone on in different degrees in different lizards. The other is that while the mass of the micro-chromosomes remains the same throughout the family, there is a tendency in different species for these bodies to fuse together into compound bodies. The latter possibility receives some support from the observation that in somatic divisions these micro-chromosomes do unite, and further, where we have a small number of micro-chromosomes, as in *Sceloporus spinosus*, they are relatively large in individual size, while when they are numerous, as in *Sceloporus U. consobrinus*, they are relatively smaller. (Compare micro-chromosomes of figs. 25, 26, 27 with small bodies in text fig. 1, A.)

Whatever the explanation be for the micro-chromosomes, it is clear that the macro-chromosomes of all the Iguanidae are all strikingly alike, and they give additional evidence for the validity of McClung's generalization which was based on a study of grasshopper chromosomes.

4. *Syncytial masses*

A very striking feature of the testes of all lizards is the common occurrence of so-called syncytial masses. Similar bodies have been reported for turtles (Jordan), birds, and mammals.

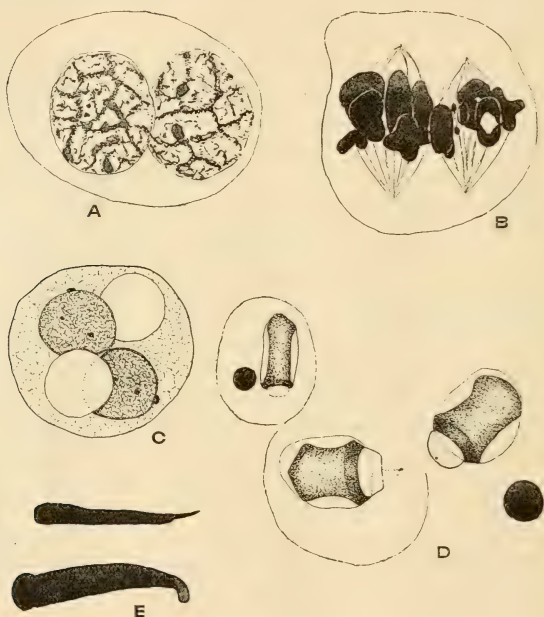
In lizards these masses are most clearly seen in well-matured individuals where the lumen of the tubule is large. In such cases germ-cells tend to scatter somewhat from the walls of the tubules, and one finds here and there rounded masses of cytoplasm containing from two to eight or more nuclei, but with no distinguishable cell walls. Typically, one finds two primary spermatocytes thus associated (text fig. 6, A). Occasionally there may be more primary spermatocyte cells. Figure B shows such a syncytial mass, as seen in A, undergoing division. Masses showing secondary spermatocyte cells have from six to eight nuclei, and many spermatids may be joined in this fashion.

The formation of these syncytial masses is probably due to the failure of the cytoplasm to divide after the chromosomes have separated. However, it does not seem to interfere with the normal formation of spermatozoa, for one finds such masses as shown in figure C where the formation of the sperm heads is going on normally.¹⁰

In passing, attention should be called to interesting theoretical possibilities which such a figure as 6, B, affords. Here are two first spermatocyte spindles lying closely associated side by side. It must be a very rigid division mechanism which would prevent at some time a chromosome from getting into the wrong spindle. This would seem to give a mechanism for variation in chromosome number, and if the sperm thus formed were viable would give

¹⁰ In this connection it is interesting to note that Jordan has found the same condition in the turtles. He describes two, three, or even four sperm arising from the same cytoplasmic mass.

interesting genetic possibilities in the resulting zygotes. The widespread occurrence of syncytial masses among mammals and birds where the same condition must exist makes the observation of some interest.



Text figure 6 A, B, and C. Syncytial masses. D and E show giant spermatozoa in process of formation.

5. *Giant sperm*

An additional feature in lizard spermatogenesis is the occurrence of giant spermatozoa. These are rare, but when found are very conspicuous. In text figure 6, D, two such giant spermatozoa and a spermatozoon of normal size are seen in the process of formation. It will be noted that the giant size affects not only the nucleus, but also the achromatic element. In figure

E a giant spermatozoon and one of normal size are seen (the tails are not indicated in the figure). The giant spermatozoa tend to occur in patches.

The author has followed in some detail the spermiogenesis in lizards. It is characterized by the elimination of enormous amounts of cytoplasm. An account of the process will not be given, however, until mitochondrial stains have been employed. At this later time it is hoped that the origin of the giant spermatozoa can be determined.

SUMMARY

1. This study on the spermatogenesis of lizards was undertaken with two points in view: first, to determine if sex-chromosomes were present and, second, to see what light could be thrown on the peculiar condition found by Guyer in the spermatogenesis of birds.

2. Seven species of lizards, including two families, were examined and their spermatogenesis reported on in this paper.

3. In *Anolis carolinensis* there are twelve large V-shaped chromosomes (which for convenience have been termed macro-chromosomes) and about twenty-two small dot-like chromosomes (called micro-chromosomes) in the spermatogonial cells. In the primary spermatocytes there are six macro-chromosomes and eleven micro-chromosomes. One of these macro-chromosomes is bipartite in form and passes to one pole of the cell undivided. As a result, the second spermatocytes are of two kinds, part with five and part with six macro-chromosomes. The sperm are dimorphic as regards this bipartite chromosome, which has been identified as the sex-chromosome. As far as could be determined, the small micro-chromosomes were equally distributed in both of the maturation divisions.

4. The spermatogenesis of *Sceloporus spinosus* was found to be essentially like that of *Anolis*. There are twelve macro-chromosomes and ten micro-chromosomes in spermatogonia. In first spermatocytes there are six and five of these bodies, respectively. The X-chromosomes are frequently seen passing

early to one pole of the cell, undivided. As a result, the sperm are dimorphic as regards the sex-chromosome.

5. The spermatogonia of *Sceloporus undulatus* V. consobrinus show twelve macro- and about eighteen micro-chromosomes. An X-element is present, and behaves as it does in *Anolis* and *S. spinosus*.

6. The spermatogenesis of *Holbrookia texana* is essentially like that of the foregoing forms. There are twelve macro- and twenty-two micro-chromosomes in the spermatogonia. An X-element was found, and the sperm are dimorphic as regards it.

7. The spermatogenesis of *Uta ornata* was only partly worked out. There are twelve macro- and about eighteen micro-chromosomes in the spermatogonia. While the X-element was not identified in the first maturation division, the second spermatocytes were found to be of two kinds, part with five and part with six macro-chromosomes. Hence this species is like the rest described.

8. The chromosome complex of *Crotaphytus collaris* was determined. There are twelve macro- and from twenty-four to twenty-six micro-chromosomes in the spermatogonia. The primary spermatocytes show a halving of these numbers.

9. A fragmentary description of the spermatogenesis of *Cnemidophorus gularis* is given. This species belongs to a different family from the rest which were worked upon.

10. Sex-determination in lizards is of the 'double accessory' type; that is, the X-chromosome is derived from two spermatogonial chromosomes. The males are heterozygous as regards sex.

11. A study of ovarian tissue in *Sceloporus spinosus* indicated that the female was of the 2X condition, for two more macro-chromosomes were found present than in the males.

12. Dividing somatic cells of *Sceloporus* embryos show either twelve or fourteen V-shaped macro-chromosomes. The number is constant for the individual; that is, we find always the same number in a given embryo. The embryos with twelve macro-chromosomes would have become males and the ones with four-

teen macro-chromosomes would have become females, without doubt.

13. There is a remarkable constancy in the number and size of the macro-chromosomes of all the species of the family Iguanidae studied. All six species reported on show twelve macro-chromosomes in dividing spermatogonia, and in every case at least three pairs of the chromosomes are strikingly alike in size and shape.

14. Syncytial masses and giant spermatozoa are commonly found in the testes of all the lizards studied.

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PLATES

All figures were drawn with the aid of a camera lucida, a B. & L., $\frac{1}{12}$ oil immersion, and a no. 15 eye-piece. The microscope was elevated on a stand, so that all figures in this paper represent a magnification of approximately 5000 diameters, which has been reduced one-third in reproduction.

PLATE 1

EXPLANATION OF FIGURES

All drawings in this plate were taken from the testis of *Anolis carolinensis*.
1 to 3 Are spermatogonial equatorial plates. The letters refer to chromosomes which can be identified.

4 A very young first spermatocyte, showing two nucleoli.

5 Diplotene stage, showing X-chromosome.

6 Early prophase of the first division, showing tetrads.

7 and 8 Equatorial plates of the first division.

9 to 12 Side views of the first division spindle, showing the X-chromosome passing undivided to one pole of the cell.

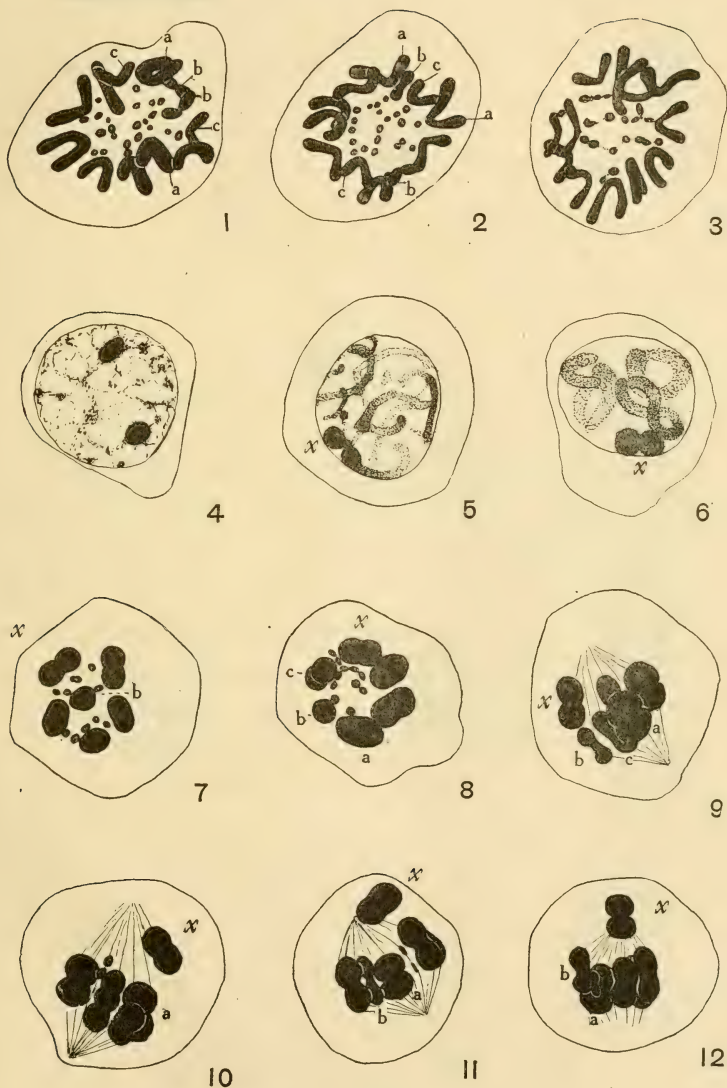


PLATE 2

EXPLANATION OF FIGURES

All figures were taken from the testis of *Anolis carolinensis*.

13 and 14 Side views of the first maturation spindle, showing the X-chromosome.

15 to 17 Late anaphase stages of the first maturation division. In the case of figure 17, the two plates overlapped, so in order to show each end clearly the slide was moved a trifle after the upper pole was drawn.

18 Side view of the second maturation spindle, showing the precocious splitting of the chromosomes.

19 Equatorial plate view of a second spermatocyte cell, showing five large chromosomes.

20 Equatorial plate view of a second spermatocyte cell, showing five large chromosomes.

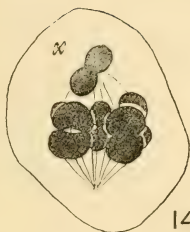
21 Late anaphase of a second spermatocyte cell, showing that five large chromosomes are going to each pole.

22 and 23 Equatorial plate views of second spermatocyte cells, showing the presence of six large chromosomes.

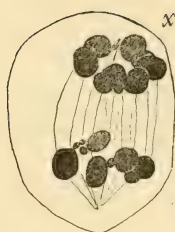
24 Late anaphase of a second spermatocyte division, showing six large chromosomes going to each pole.



13



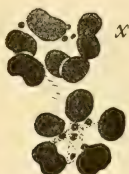
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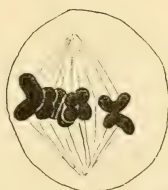
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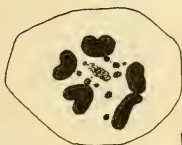
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PLATE 3

EXPLANATION OF FIGURES

All figures in this plate were taken from the testis of *Sceloporus spinosus*.

25 to 27 Equatorial plate views of spermatogonial divisions.

28 to 30 Equatorial plate views of the first spermatocyte spindle.

31 to 33 Side views of the first spermatocyte spindles, showing the early migration of the X-chromosome to one pole of the cell.

34 to 36 Equatorial plate views of second spermatocyte spindles, showing either five or six large chromosomes.

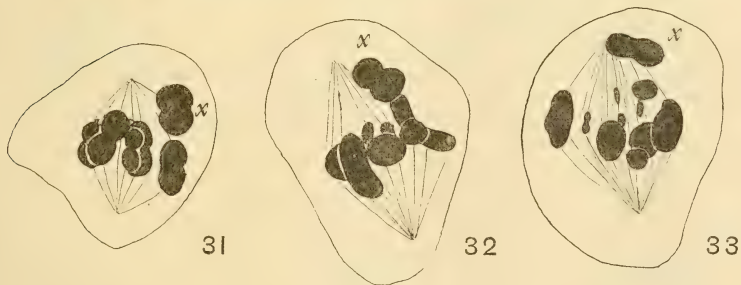
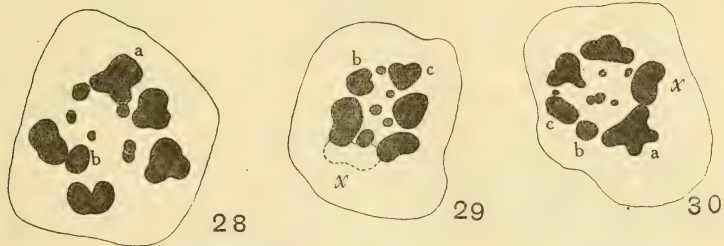
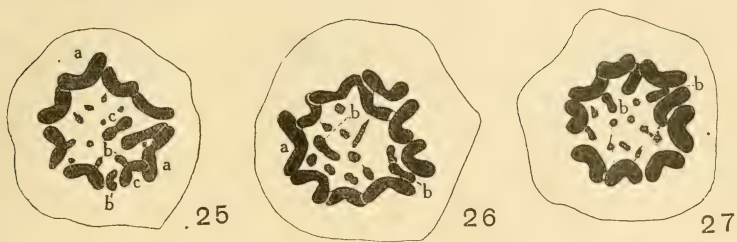


PLATE 4

EXPLANATION OF FIGURES

The figures of this plate were drawn from cells found in the testis of *Holbrookia texana*.

37 Equatorial plate view of a dividing spermatogonia.

38 and 39 Equatorial plate views of first spermatocyte spindles.

40 Side view of a first spermatocyte spindle.

41 Telophase of the first division.

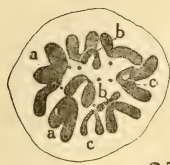
42 and 43 Late anaphases of the first division, showing five chromosomes (large) at one pole and six large chromosomes at the other pole.

44 Side view of a second spermatocyte spindle, showing the precocious splitting of the chromosomes.

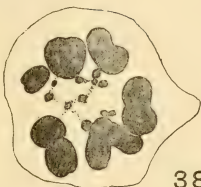
45 and 46 Equatorial plate views of the second division, showing, respectively, five and six large chromosomes.

47 Late anaphase of the second division, showing five large chromosomes going to each spermatid.

48 Late anaphase of the second division, showing six large chromosomes going to each spermatid.



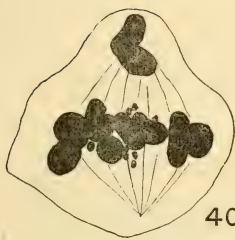
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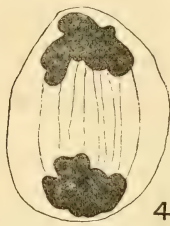
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Resumen por la autora, Lorande L. Woodruff.

Las razas micronucleadas y amiconucleadas de los Infusorios.

La autora da a conocer en el presente trabajo su descubrimiento de razas amiconucleadas de *Oxytricha fallax*, *Urostyla grandis* y *Paramecium caudatum*, discutiendo la función del micronúcleo y macronúcleo. También se ocupa del origen posible de las razas amiconucleadas, estudiando además los anfinúcleos.

Translation by José F. Nonidez
Cornell Medical College, New York

MICRONUCLEATE AND AMICRONUCLEATE RACES OF INFUSORIA

LORANDE LOSS WOODRUFF

Osborn Zoölogical Laboratory, Yale University

FOUR FIGURES

The dimorphic condition of the nuclear apparatus of the Infusoria is one of the most interesting specializations exhibited by the group, representing, as is well-known, a segregation of the so-called 'somatic' or trophochromatin from the 'generative' or idiochromatin into distinct bodies, the macronucleus and micronucleus. It has generally been accepted that this differentiation of the nucleus is a diagnostic character of the Infusoria, and, aside from parasitic or aberrant species, the only apparent exceptions have revealed the micronuclei as distinct bodies within the macronuclear membrane during vegetative stages, or emerging as such at the onset of conjugation.

It is true that there are many ciliates in which the micronucleus has not been observed, but naturally such cases are attributed rather to the difficulties sometimes involved in determining these bodies than to their absence. This conclusion has been strengthened by the fact that in micronucleate forms the removal of the micronucleus through degeneration or by experimental means has proved to be the death warrant of the cell. All the evidence indicates that once the chromatin differentiation is established during conjugation or endomixis, the die is cast so far as the macronucleus is concerned. It is destined to play its part during the vegetative life of the cell and to be replaced from the micronuclear complex at the next conjugation or endomixis period.

The recent work of Dawson¹ in this laboratory, however, has reopened one phase of the question. He found in an infusion a race of *Oxytricha hymenostoma* without a micronucleus and bred it successfully in pedigree cultures for several years. His experiments showed that periodically the animals were in a condition suggestive of conjugation, but merely abortive results followed, such as 'cannibalism,' etc. In brief, Dawson's work demonstrates that a morphologically differentiated micronucleus is not a *sine qua non* for the life of the individual free-living cell nor for the life of the race during reproduction by division. And, further, it emphasizes the irreversibility of the transformation of micronucleus into macronucleus, since, in his long series of experiments, during the stress, so to speak, of what must be interpreted as abortive attempts to conjugate, micronuclei were not forthcoming.

More evidence indicating the sufficiency of the uninucleate condition for the vegetative life of the cell has just been reported by Landis and by Patten.² The former has under culture an amiconucleate race of *Paramecium caudatum*. Furthermore, he believes that this race can conjugate successfully, and if this proves to be true it will raise a still more interesting question. Miss Patten, of this laboratory, is studying a pedigree culture of an amiconucleate race of *Didinium nasutum* which arose from conjugating micronucleate individuals.

The purpose of the present paper is to record the discovery of other amiconucleate races, representing *Oxytricha fallax*, *Urostyla grandis*, and *Paramecium caudatum*.

With regard to the technique employed, it will suffice to say that it has involved the use of the several well-established methods for the cytological study of the Infusoria, including those used in our studies on endomixis.³ Certainly, the results

¹ J. A. Dawson, An experimental study of an amiconucleate *Oxytricha*. Jour. Exp. Zool., 1919, vol. 29, p. 473; 1920, vol. 30, p. 129.

² E. M. Landis, An amiconucleate race of *Paramecium caudatum*. Amer. Naturalist, 1920, vol. 54, p. 453. M. W. Patten, The life-history of an amiconucleate race of *Didinium nasutum*. Proc. Soc. Exp. Biol. and Med., 1921, vol. 18, p. 188.

³ L. L. Woodruff and R. Erdmann, A normal periodic reorganization process without cell fusion in *Paramecium*. Jour. Exp. Zool., 1914, vol. 17, p. 425.

are consistent, since, for example, the study of two races of the same species under identical conditions invariably revealed one micronucleate and the other amiconucleate.⁴

OXYTRICHA FALLAX

A specimen of *Oxytricha fallax* was isolated from a laboratory aquarium on January 28, 1920, and a pedigree culture started by the usual daily isolation method. Standard beef extract was employed as the culture medium.⁵ A cytological study disclosed no micronuclei, and therefore the culture was not employed for the purpose intended, but carried on for a thorough examination of its nuclear apparatus and its life-history.

The graph of the rate of reproduction of the lines of this culture is given in figure 1. It exhibits no unusual features, except possibly greater fluctuation in division rate than one usually finds in hypotrichous ciliates when bred under essentially constant culture conditions. The great fall in the rate of division during the fifth five-day period, however, is clearly related to the fact that during the previous five days daily isolations were not made and fresh culture medium was not supplied. The cause of the sudden death of the race on May 4, 1920, at the 246th generation is not apparent, since the vitality as measured by division rate of the previous five-day period was not low. Twice during the life of the culture a few pairs of conjugants were observed in the mass cultures formed of animals discarded from the pedigreed lines at the daily isolations. The conjugants failed to live when isolated, and when preserved revealed no evidence of micronuclei.

In a word, this race consistently failed to reveal a morphologically differentiated micronucleus, though it possessed the potentiality to produce by division at least the number of descendants represented by 2 to the 246th power.

⁴ Miss Hope Spencer, of this laboratory, has assisted me in conducting the various pedigree lines and in making permanent preparations. Professor Bait ell oriented, by his plasma method (Proc. Soc. Exp. Biol. and Med., 1921, vol. 18, p. 172), certain animals for sectioning.

⁵ L. L. Woodruff and G. A. Baitsell, The reproduction of *Paramecium aurelia* in a constant culture medium of beef extract. Jour. Exp. Zool., 1911, vol. 11, p. 135.

UROSTYLA GRANDIS

On May 4, 1920, a heavy growth of *Urostyla grandis* was discovered in a laboratory aquarium, and during the following week ten animals were isolated to form ten pedigree lines. These were bred on the standard beef-extract medium which was supplied fresh daily and inoculated with a pure culture of *Colpi-*

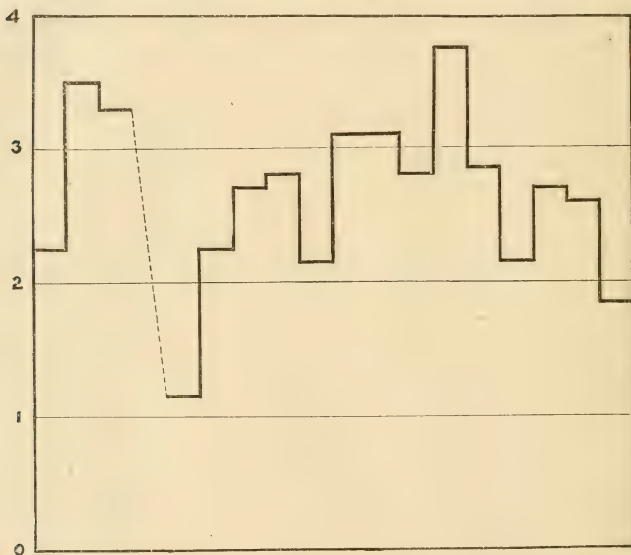


Fig. 1 Graph of the rate of reproduction of an amiconucleate race of *Oxytricha fallax*. The average daily rate of division of the lines is again averaged for five-day periods.

dium. *Urostyla* will survive on the bacterial flora which normally develops in the beef extract, but for vigorous growth some small protozoan form must be supplied as food.

Four of the pedigree lines of *Urostyla* proved to be micronucleate and six, amiconucleate. Accordingly, they were not suitable for the experiments planned, but were continued for varying periods for further cytological study. The ten lines are summarized in table 1.

The graph of the division rate of line Ub which was continued longer than any of the others and accordingly studied most thoroughly is given in figure 2.

TABLE 1

LINE	MICRONUCLEUS	DAYS BRED	GENERATIONS ATTAINED
Ua	Absent	80	40
Ub	Absent	191	128
Uc	Present	22	8
Ud	Absent	33	15
Ue	Present	4	3
Uf	Present	18	9
Ug	Absent	3	2
Uh	Absent	35	10
Ui	Absent	3	2
Uj	Present	27	19

In brief, these ten lines throughout their life bred true with respect to the presence or absence of micronuclei—certain lines (Uc, Ue, Uf, Uj) invariably exhibiting large, easily discernible micronuclei, while other lines (Ua, Ub, Ud, Ug, Uh, Ui) consistently were without micronuclei (cf. figs. 3 and 4).

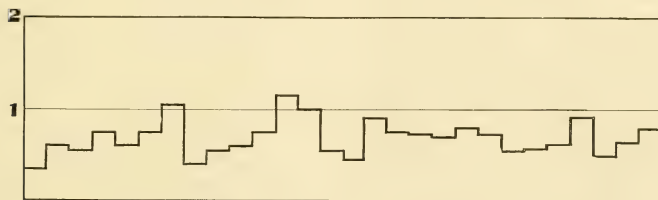


Fig. 2 Graph of the rate of reproduction of an amiconucleate race of *Urostyla grandis*. The average daily rate of division of the lines is again averaged for five-day periods.

Urostyla grandis is not a common hypotrichous form, and the only time I have ever found it at New Haven was in the mass culture mentioned above. Accordingly, it seems probable that all of the animals of the 'wild' culture were descended from the same stock and that the separation into a micronucleate

and an amiconucleate race occurred while in this culture. If such was the case, it is reasonable to suppose that the six amiconucleate individuals isolated to start the six amiconucleate lines were descended in the 'wild' state from a single animal, and likewise all the micronucleate animals were the progeny of



Fig. 3 *Urostyla grandis*. Micronucleate race, line Uc, 4th generation, May 14, 1920.

Fig. 4 *Urostyla grandis*. Amiconucleate race, line Ud, 8th generation, May 18, 1920.

a single animal. If so, then it was merely a matter of chance that four micronucleate and six amiconucleate lines were isolated from the 'wild' culture. At all events, it is highly interesting that the *Urostyla* population of the 'wild' culture exhibited two distinct racial types with respect to their nuclear apparatus.

PARAMECIUM CAUDATUM

During January, 1920, the author's course in protozoology made a study of the cytology of *Paramecium aurelia* and *Paramecium caudatum* and incidentally discovered a race which had the usual characteristics of *P. caudatum* except that no micronucleus could be demonstrated. It seems justifiable to conclude that a micronucleus was absent, though the race was not studied for a long period in pedigree culture. Apparently it was a race comparable to that recently discovered by Landis.

DISCUSSION

The facts in regard to amiconucleate races of ciliates are as yet too few to warrant an attempt to discuss from in a comprehensive way the questions which they present, though a brief survey and interpretation will serve to delineate the problem.

Confining attention to free-living ciliates, we find five species in which amiconucleate races have been definitely announced, viz., *Oxytricha hymenostoma* (Dawson), *Oxytricha fallax* (Woodruff), *Urostyla grandis* (Woodruff), *Paramecium caudatum* (Landis, Woodruff), and *Didinium nasutum* (Patten). To extend the survey to the general literature on the morphology of ciliates is not fruitful, because, unless the micronucleus was the special point of study, the evidence is of little value. Two records, however, may be mentioned which are highly significant. Thon, in 1905,⁶ made a careful cytological study of *Didinium*, but was unable to discover a micronucleus, and Miss Moody, in 1912,⁷ carried on a long pedigree culture of *Spathidium spathula* which revealed no micronuclei. It is difficult to conceive that either of these authors could have overlooked micronuclei if present. The micronuclei of *Didinium*, though small, are usually fairly easy to demonstrate when present. The micronuclei of *Spathidium* were clearly observed by Maupas,⁸

⁶ K. Thon, Ueber den feineren Bau von *Didinium nasutum*. Archiv f. Protistenk., 1905, Bd. 5, S. 282.

⁷ J. E. Moody, Observations on the life-history of two rare ciliates, *Spathidium spathula* and *Actinobolus radians*. Jour. Morph., 1912, vol. 23, p. 349.

⁸ E. Maupas, Sur la multiplication des Infusoires ciliés. Arch. d. Zool. Expér. et Génér., 1888, 2^e Série, T. 6, p. 247.

and are present in a pedigree race of this organism which the present writer now has under cultivation.⁹ It is also significant that Miss Moody's race of *Spathidium* could not be induced to conjugate, whereas the writer's culture conjugates successfully at frequent intervals.¹⁰

The evidence at hand certainly shows that races of free-living ciliates exist which do not exhibit the orthodox nuclear apparatus. The evidence also indicates that amiconucleate forms are either unable to conjugate or, if cell fusion does occur, the result is abortive. It is certain that a morphologically defined micronucleus is not necessary for the continued existence by vegetative division of certain races of common free-living ciliates which typically have this cell organ.

If, on theoretical grounds, one is predisposed to believe that the presence of 'germinal' chromatin (ordinarily segregated in a micronucleus) is necessary for the life of the cell, then, since amiconucleate cells live and divide, the nucleus which is present must be regarded not as a macronucleus in *sensu stricto*, but as an amphinucleus. If this interpretation is correct, the relation between idio- and trophochromatin in the amphinucleus is apparently of such a nature that the former is unavailable for the conjugation phenomena, since the evidence to date indicates that conjugation either is not attempted or is abortive in amiconucleate races.

It is possible that amiconucleate races arise by the transformation of all the micronuclei, resulting from the reconstruction micronuclear divisions after conjugation or endomixis, into macronuclei. Such a result was observed by Prandtl in his study of the cytology of conjugation in *Didinium*, though he doubted if the animals with a nuclear heritage of this character were viable.¹¹

⁹ L. L. Woodruff and Hope Spencer, The structure and behavior of *Spathidium* spathula with special reference to the capture and ingestion of its prey. *Jour. Exp. Zool.* In press.

¹⁰ L. L. Woodruff and Hope Spencer, The early effects of conjugation on the division rate of *Spathidium*. *Proc. Soc. for Exp. Biol. and Med.*, 1921, vol. 18. The survival value of conjugation in the life history of *Spathidium*, *Ibid.*, 1921, vol. 18.

¹¹ H. Prandtl, Die Konjugation von *Didinium nasutum*. *Archiv f. Protistenk.*, 1906, Bd. 7, S. 251.

A concrete case will serve to make the point clear. In *Paramecium caudatum*, according to Calkins and Cull, the syncaryon divides three times to form eight micronuclei, four of which become transformed into macronuclei and four remain micronuclei. Since four micronuclei remain micronuclei, the differential which is at the basis of their persistence presumably was instituted or became effective at the third micronuclear division—the division which segregated idiochromatin from trophochromatin. Calkins and Cull write:

We have been unable to tell from morphological data which of the first eight somatic nuclei are destined to form four macronuclei; so far as we can tell there is no difference in the chromatin of these nuclei until that swelling begins which characterizes the young macronucleus. . . . Here differentiation takes place, therefore, first by nuclear division and second by chromatin metamorphosis, and once transformed. . . . the macronucleus is ever after a macronucleus until its ultimate dissolution.¹²

Now a cell which received, in the normal course of reconstruction, such a micronucleus and macronucleus would be a typical animal—one in which the idiochromatin and trophochromatin was segregated into distinct bodies. Such an animal would represent the type which experimental work has shown to be, as a rule, non-viable in the absence of a micronucleus.

On the other hand, it is possible—perhaps probable when we recall the observations of Prandtl and of Patten—that under certain exigencies during cell reorganization the typical course of events may be disturbed so that the differential micronuclear division is suppressed or not precise and all the micronuclei metamorphose into 'macronuclei.' Such 'macronuclei' may be interpreted as amphinuclei. There is, a priori, no reason why nuclei of this character should not be adequate for all the typical life phenomena of the cell, except probably those involving endomixis or conjugation. Such apparently is the potentiality of the nuclear apparatus of the amiconucleate races thus far described.

¹² G. N. Calkins and S. W. Cull, The conjugation of *Paramecium caudatum*. Archiv f. Protistenk., 1907, Bd. 10, S. 406-407.

Resumen por el autor, Hoyt S. Hopkins.

Las condiciones para la conjugación en las diversas razas de *Paramecium*.

El autor demuestra mediante un estudio comparativo de más de veinte líneas genealógicas de *Paramecium* que las condiciones necesarias para la conjugación no son necesariamente las mismas en todas las razas de una especie determinada. En este respecto existen marcadas diferencias en las diversas razas de *P. caudatum*. Algunas se conjugan fácilmente bajo las condiciones ordinarias del laboratorio, mientras que otras en las mismas condiciones, o no se conjugan o lo hacen raras veces. Los experimentos más eficaces en la producción de la conjugación en una línea genética determinada pueden ser ineficaces en otra. Diferencias semejantes, pero menos marcadas, existen entre las razas de *P. aurelia*. El autor considera el papel desempeñado por el "adormecimiento" o periodo de divisiones infrecuentes mantenido durante largo tiempo en su relación con el proceso de la conjugación. Fundándose en el hecho de que los animales sometidos al adormecimiento pueden hacerse conjuguar más fácilmente y son capaces de divisiones más rápidas que los animales que se han multiplicado previamente, el autor indica que la división libre puede actuar como un estímulo primario para la conjugación. Esta relación es más aparente por el conocimiento de los efectos de ciertas soluciones salinas que favorecen la conjugación y que también actúan como estímulos de la división. Las "periodicidades" observadas en las líneas genéticas en conjugación están relacionadas con fluctuaciones periódicas de la rapidez de la división; el intervalo de tiempo entre cada periodo de conjugación está tal vez condicionado por la fase de división reducida que sigue a la conjugación. De estas consideraciones se desprende que, al parecer, la conjugación de *Paramecium* está determinada por la acción combinada de factores externos e internos, aunque la relativa importancia de cada uno puede ser diferente en diversas líneas genéticas.

THE CONDITIONS FOR CONJUGATION IN DIVERSE RACES OF PARAMECIUM

HOYT S. HOPKINS

Zoological Laboratory of the Johns Hopkins University

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INTRODUCTION

Maupas ('89) was one of the first to suggest differences between nearly related species of Infusoria in respect to their tendency to conjugate. He found, for example, that *Colpidium colpoda* would conjugate more readily than the similar species *C. truncatum*. Like differences have been shown by later investigators to obtain in other genera, as in the two species of *Colpoda* studied by Enriques ('08). It had long been known, too, that cultures of the same species differ much as regards susceptibility. Most authors attributed these differences to environmental factors, as mixed cultures of uncertain derivation were used in most cases. As pure, or 'pedigreed,' races began to be used, these cultural diversities were conceived of as having possibly a racial basis. If diversities with respect to size, shape, and division rate have their origin in heredity, may it not be that differences in the ability to conjugate are based upon hereditary factors?

This view has led to some controversy in recent years in regard to the existence of so-called 'non-conjugating races,' calling into question the universality of fertilization among the Infusoria.

That the process may be postponed indefinitely in *Paramecium aurelia*, provided the cultural conditions are kept uniform and favorable, seems probable in the light of Woodruff's extended work, for he maintained one strain through more than six thousand generations without conjugation (1917). This same race of organisms was subjected to conditions which favor conjugation with positive results (Woodruff, '14). It would appear from these results that, in *Par. aurelia* at least, the occurrence of conjugation is facultative under uniform conditions of environment. Other reports, moreover, would indicate that conjugation is possibly lacking in the life-history of some races of *Paramecium*, or recurrent only after very long time intervals in others. Thus Jennings ('10) maintains that there exist in these organisms striking racial differences with respect to conjugation. In some of the strains employed in his work normal 'epidemics' occurred frequently, sometimes apparently under uniform conditions of existence, in some only infrequently, and in others not at all. He emphasized also growth and nutrition as important factors in regulating conjugation. Thus a period of rapid multiplication followed by a decline in the division rate usually gave an epidemic of conjugation in the majority of cases. A preliminary period of semistarvation (reduction in division rate) of two weeks or longer made the organisms more susceptible when the above-mentioned procedure was followed. Some few cultures (races) failed to yield conjugants even when the most favorable conditions were afforded. Two species of *Paramecium* (*caudatum* and *aurelia*) were used in his work, and the same kind of differences was found in each: there were races which conjugated freely, often at frequent intervals, others which did not, although *P. caudatum* showed less frequent epidemics and was characterized by a greater number of the 'non-conjugating' races.

The process of nuclear reorganization, or endomixis, discovered by Hertwig ('89), and worked out by Woodruff and Erdmann ('14, '16), would seem to suffice in maintaining the normal vegetative processes in *Paramecium* in the entire absence of conjugation. Even in such a form as the hypotrichate *Uropeltus*, according to the work of Calkins ('19, two papers),

conjugation is probably not essential to the continued life of the race. For, although a nuclear reorganization process occurs only within the cyst, animals can be derived from the cysts which develop with the same renewed vigor as characterizes exconjugants.

The direct effect of altered environmental conditions in determining conjugation was more strongly emphasized by Enriques ('09), who employed solutions of electrolytes to induce epidemics of conjugation in *Cryptochilum nigricans*. His studies led him to believe that the process is in no way bound up with an inferred 'life-cycle,' and so is not dependent primarily upon internal factors. This same general method was adopted later by Zweibaum ('12) in a careful study of a race of *Paramecium caudatum*. During the early history of this race conjugation could be induced at times simply by subjecting samples of the flourishing cultures to the action of certain salt solutions. Later it was found necessary to subject the cultures to a preliminary period of partial starvation ('disette') lasting from five to six weeks. Cultures which had been maintained under conditions of steady growth ('cultures continuatives') no longer gave conjugants when treated with salts, except in rare instances, and then in very small numbers. From the 'dormant' cultures, however, conjugants were readily obtained after allowing the organisms to multiply rapidly in a rich culture medium, changed every three or four days during a period of about one week, then subjecting them to the action of the salt solutions in optimal concentrations (as determined previously by experiment). This last process is to be carried out with large numbers of paramecia, in a small culture dish, the organisms being thus limited to a small amount of nutriment. The solutions of salts are then added in the ratio of three parts of salt solution to one part of culture (15 cc.: 5 cc.). The essential steps in the procedure are seen to be those laid down by Maupas—with an added factor, namely, the treatment with a chemical reagent. In general the chlorides of aluminium, gold and ferric iron, and the salts of sodium, were found to be most effective. Just what rôle the electrolytes may serve, whether as an additional stimulant or in some other

more specific way, is not clear. Hypertonic solutions of 'tap-water' or of glucose produce like results to a lesser degree, but they act apparently by increasing the osmotic concentration of the medium, so that their effect is probably not a specific one in the chemical sense. The result produced by any one of these reagents is to augment the intensity of conjugation (increase the proportion of conjugating animals), for a small percentage of individuals may conjugate in a culture to which water alone has been added (in place of salt solution), or sometimes even in the main culture which had been renewed with hay infusion.

In the light of this recent work in which refined methods of experimentation were employed successfully with one race, the question has been asked whether we cannot by such means induce conjugation in any race of infusoria. As applied to *Paramecium* in particular—are there any racial differences in respect to conjugation, or are the alleged diversities environmental in origin? According to Zweibaum, we ought to be able to induce conjugation in any race of *Paramecium caudatum* by using those methods which he finds most effective, for he holds that all supposed racial diversities, such as those described by Jennings and Hargitt ('10) for size, result from cultural differences. To state his position more clearly, I quote from page 276 of his paper:

Dernièrement encore pour les *Paramaecies* Jennings détermine diverses races—injustement à mon avis—et affirme que les conditions de conjugaison diffèrent considérablement selon ces races. L'auteur déplace ainsi le centre de gravité de la question sans ajouter une conception nouvelle et un déterminisme positif dans les considérations de conjugaison chez les *Paramaecies*.

And again, in summarizing the conditions which he considers sufficient for conjugation in *Par. caudatum*, he writes (p. 292):

Comme conclusion nous pouvons dire que les *Paramaecies* que Jennings et Hargitt croient appartenir à des races diverses, se conjugueront toujours, lorsque après avoir passé par un état de disette prolongée de 5-6 semaines, on remplira les autres conditions nécessaires—à savoir—la disette comme agent de l'action instantanée, la température de 20-23°C. et la composition du milieu (voir 2^{ème} partie).

Ce qu'on dit être des races diverses de *Paramaecium* n'est que le résultat de la quantité de la nourriture.

It is apparent from these statements that Zweibaum holds that the conditions for conjugation as worked out for one race of *Paramecium* apply to all other races of the same species—at least in *P. caudatum*; and he strongly inclines toward the view that conjugation is regulated primarily through environmental conditions. This, at any rate, is the view expressed by Enriques (on the basis of Zweibaum's work¹), for he did not see fit to change his original point of view, believing that the preliminary period of semistarvation of five to six weeks to which the paramecia were subjected was but a step in the experimental procedure, making the organisms more susceptible to the effects of the salts.

The present study was undertaken mainly with this object in view: to ascertain whether the so-called 'conditions for conjugation' worked out for one race of *Paramecium* are effective for other races and, in general, to determine so far as possible the differences in the conditions required to induce conjugation in different races. An attempt was made, also, to find out whether the various races under observation were characterized by different time intervals between conjugation periods, or, as we might say, by diverse 'sexual periodicities.' Jennings ('10) called attention to such apparent periodicities in certain races of *P. aurelia*. His studies on *P. caudatum*, while less extensive, indicated that in some races of this species conjugation occurs at intervals of several months, or perhaps years. The importance of further work relative to this point was felt more keenly in view of the greater perfection attained in the experimental (or environmental) study of conjugation. Even in other groups of organisms the study of natural periodicities in respect to sexual activity, apart from environmental influences, has not received the attention which it merits.

This work was undertaken at Dr. H. S. Jennings' suggestion, with this end in view. I wish here to express my thanks to Professor Jennings for his kindly advice and criticism, as well as for assistance in the procuring and rearing of material for cultures.

¹ See Zweibaum ('12), footnote by Enriques, pp. 346-347.

For our purpose, then, three general questions should be kept in mind: 1) Do there exist differences between various races of *Paramecium* with respect to conjugation? 2) If so, what are they? 3) From a comparative study of different races of the same species and of the different species, what can be said in general regarding the nature of the conditions for conjugation in the Infusoria?

Any answer to the third question must depend, in part at least, upon the facts brought out in connection with the other two. If constant natural differences in the time interval between successive epidemics of conjugation in different races and species can be demonstrated, it would coincide better with the view that conjugation is primarily a function of some internal factors, although, of course, it would not exclude the setting in operation of these factors by environmental conditions. The complete subordination of conjugation to direct environmental conditions might lead us to regard the process as mainly environmental in its origin, and perhaps directly adaptive in its functional significance.

MATERIAL AND METHODS

Twenty-four races of *Paramecium*, each derived originally from a distinct individual, were used in the course of this work; eleven representing *P. caudatum*, and thirteen, *P. aurelia*. Many other races were isolated, but owing to unfavorable culture conditions died out before reaching the 'mature' condition of old cultures.

This dying out of unbalanced cultures is conditioned largely by the racial characteristics of the protozoan isolated, it being ill adapted to the conditions of growth supplied in the laboratory. This is shown by the fact that other races which were reared with difficulty to the condition of stable cultures in the beginning were found to show, some months later, this same inability to develop rapidly when isolated individuals were reared again under rich culture conditions. Deleterious strains of bacteria undoubtedly serve as the immediate cause of the death of a culture in most cases, but the diversities between races of pro-

tozoans as regards resistance must be taken into account in order to understand why one succumbs in an environment in which another grows and apparently thrives. If two races distinct in size, one of *Par. caudatum*, the other of *aurelia*, are reared together in the same culture dish, hence in the same medium, one may survive, the other die, or both may live together. Like differences undoubtedly exist between races of the same species, for my work indicates that such races show constant natural differences as regards their resistance to specific reagents and their ability to grow readily in slide cultures as well as in mass cultures.

During the course of development of these cultures records were taken of races under similar growth conditions, in order to determine in advance which ones were subject to periodic epidemics of conjugation and the nature of these rhythmic changes, so as to compare their behavior under usual conditions with that to be obtained later in the experiments. Certain cultures were subjected to daily observation for weeks at a time with this object in view, and record kept of sporadic occurrences and of conjugation *en masse*. This revealed what races were subject to frequent epidemics and in what ones conjugation was rare or lacking. Most of these observations were made daily under a binocular microscope of wide field of vision, upon cultures growing in Stender dishes measuring 5 cm., 6 cm., and 10 cm. in diameter, so that any but rare conjugating pairs could hardly be overlooked. The same method of daily observation was adopted during the course of experiments with small cultures, and since these cultures were comparatively free from sediment, it was an easy matter to conduct thorough observations upon them during the few days required for each experiment.

Many of the experiments referred to in this paper were carried out after the procedure recommended by Zweibaum (p. 341), using salt solutions, with the object of showing whether each race would not yield conjugants when the conditions which he found most favorable were supplied. For this purpose subcultures were derived from the main cultures by diluting 50 to 100 cc. of the culture containing many *paramecia* with an equal

part of distilled water. These were allowed to stand for several weeks as 'dormant cultures,' with very little nutriment, at the end of which time hay infusion was added. Such cultures to which hay infusion has been added to induce rapid multiplication will be referred to as 'renewed cultures.' After about the fifth day portions of these cultures were treated with salt solutions in small Stender dishes.

The renewed cultures of *P. aurelia* often yielded about as many conjugating pairs as the smaller (experimental) cultures derived from them, and treated with salt solutions. It was found further that certain inorganic salts added to the renewed cultures directly, i.e., immediately following the period of dormancy, at the time of renewal, made these organisms (*P. aurelia*) more susceptible to conjugation after the preliminary period of multiplication of about five days. Small watch-glass cultures derived from these usually yielded conjugants earlier—after the third or fourth day following renewal of the main culture—but the renewed cultures themselves often yielded just as many pairs after the fifth day, showing that the sudden change brought about through such 'isolation experiments' is not an essential condition for the appearance of conjugation.

In general it may be said that large cultures of *Paramecium*, of either species, give fewer conjugating pairs than small cultures of the same race when treated in a similar manner. This condition may perhaps be attributed to the greater accumulation of organic matter (excretion products) in large cultures than in small and in part to the more uniform conditions.

The experimental procedure will be described in greater detail in connection with the experiments themselves. The culture medium used throughout during the course of this work on *Paramecium* consisted of hay infusion. 'Tap-water' was used to maintain the stock cultures, which were contained in battery jars and kept at room temperature. Cultures intended for use in future experiments were diluted with distilled water just before being subjected to their period of dormancy, and at the end of this period were renewed with an equal part of hay infusion in distilled water, changed subsequently every three to four days.

So after the multiplication period of five days or longer the organisms to be subjected to the treatment with salts were in essentially a distilled water medium. Spring water from a perpetual spring near the Johns Hopkins University campus was found to be most favorable for maintaining *Paramecium* lines on depression-slides (as slide cultures), for the organisms are very sensitive to sudden changes in the character of the medium such as attend this method.

The various strains of distinct origin are designated each by a separate number, usually followed by a small letter. Cultures of the same strain or race derived each from one individual of the original culture (frequently from an exconjugant), are designated by the same number followed by a different letter. Subcultures are often distinguished by Roman numerals.

1. CONJUGATION IN DIVERSE STRAINS

a. Paramecium caudatum

I shall take up first the question of conjugation in races of *P. caudatum*, because it was in a race of this species that Zweibaum worked out the conditions which he claims to be equally applicable to any race of this species. For, if his conclusion is valid, it should be possible to repeat his experiments upon various other strains under parallel conditions, and thus show definitely that there are no inherent diversities with respect to conjugation. On the other hand, if two races which have had the same environmental history, react differently under these same experimental conditions, one giving conjugants, the other not—or even if they react differently to other conditions which have been shown to favor conjugation in some one race—then we have evidence for racial diversities.

The following series of experiments with three races of *P. caudatum* are illustrative of others which were carried out at other times with the same races under less parallel conditions. Experiments with salt solutions found by Zweibaum to be most effective in his work were conducted upon each strain on the same days (table 1). Cultures *6aII* and *5aII* had been dormant since

March 28, 1919. A culture, *17aII* (containing also individuals of a race of *P. aurelia*, *13a*), had been dormant since April 5th. On May 8th the first of these cultures was renewed, and on the following day the other two cultures. Experiments were set on May 13th from samples of each culture, using NaNO_3 and FeCl_3 solutions; and these gave, in the case of *5aII* and *6aII* no conjugants, in *17aII*, however, conjugation to the extent of 30 to 50 per cent (May 14th). Conjugants were found in smaller numbers (2 to 5 per cent) in the renewed culture of *17aII* to to which no salts had been added, so that the results obtained with this race are clearly comparable to those obtained by Zweibaum in his experiments: the effect of the salts is to augment the intensity of conjugation.

This same race had shown its ability earlier to yield conjugants, when another culture, *17aI*, was renewed after about ten days of semistarvation (conjugation on February 26th). Similar results were obtained with a third culture of this race, *17aIII*, on April 10th and 11th, when renewed after fifteen days of dormancy. Other cultures of *17aIII* (containing also individuals of *P. aurelia*) likewise gave conjugation (table 2).

That the negative results obtained in the above experiments with cultures *5aII* and *6aII* cannot have been accidental (resulting perhaps from unfavorable culture conditions) seems certain from the fact that similar experiments upon other portions of these cultures, renewed after longer or shorter periods of dormancy, all proved negative (table 1). The further history of these two races may also be referred to in support of this view. Both were isolated from the same abandoned hay culture on February 2, 1919, and were grown under similar conditions from the start (i.e., as stock cultures). No sign of conjugation was observed in either race during the first six months of their history, although frequent attempts were made to bring it about by means of isolation experiments, as well as through the experiments with salts described above. The main cultures were allowed to go dormant from about the middle of July until October 15, 1919, on which day they were renewed. On the twentieth a few conjugating pairs (three or four) were observed in *6a*, and

again on the twenty-second. Not one was observed in *5a*. Attempts were made (on October 17th and October 22nd) to induce the occurrence of conjugation in large numbers by subjecting subcultures to the action of 0.00002 N FeCl_3 and of 0.001 N NaNO_3 solutions, but the results were negative. It should be remembered, however, that these animals taken from stock cultures had been growing in a medium made up with 'tap-water,' and so perhaps might not have responded in the same way as animals in a pure aqueous medium when treated with salts. These same stock cultures were then allowed to undergo another long period of dormancy (which may be dated from November 1, 1919). Two subcultures taken from these stock cultures and renewed with spring water and hay infusion on December 12th gave, in the case of *5a*, one pair of conjugants, in *6a*, a considerable epidemic of conjugation (1 to 5 per cent) extending over a period of thirty-three days. Attempts to augment the intensity of conjugation in the latter culture by treating samples with different concentrations of FeCl_3 (0.00002 N, 0.00004 N, and 0.00008 N), with KCl (0.00025 N and 0.0005 N) and with NaNO_3 (0.001 N) were ineffective; that is, the relative number of pairs obtained in these experiments was not greater than in the renewed culture.

A quite different state of affairs was found to ensue during the later history of *17a*. As pointed out above, this race proved susceptible to conjugation during its early development. Cultures *17aI*, *II*, and *III* taken at different times from the same stock culture (which was started on January 17, 1919, from one individual) yielded conjugants very readily when renewed after two or three weeks of semistarvation (tables 1 and 2). The general effect of adding salts to the medium was to intensify conjugation in accordance with the results of Enriques and Zwi-
baum. After four months of cultivation, however, no more conjugants were obtained in the experiments with this race. The culture *17aV*, derived from *17aIII* (which had been continued as the stock culture), when renewed on June 28th after lying dormant for one month, gave no conjugants, and experiments with salt solutions (FeCl_3 and NaNO_3), such as were

found to be effective in earlier experiments with this race, gave negative results. A subculture, taken from the main culture *17aIII*, after it had lain dormant for two months, when renewed on September 19th, gave only one pair of conjugants. The main culture itself (*17aIII*), renewed on October 15th, after three months of dormancy, gave no conjugants, although samples were treated, as in the case of *5a* and *6a* (p. 349), with solutions of FeCl_3 (0.00002 N) and NaNO_3 (0.001 N).

Another set of experiments was conducted with these three races, after more than a year of cultivation, during the month of March, 1920. A subculture was derived from each of the stock cultures, *17aIII*, *6aI*, and *5aI*, which had been maintained in a dormant state since October, 1919, and these were renewed on March 18th with distilled water and hay infusion. A good growth of paramecia followed. Daily examination of the renewed cultures revealed no conjugants in *17aIII*, a single pair in *6aI* (on March 25th), and about 1 to 5 per cent in *5aI* between March 22nd and 27th. The race *17a* is thus shown to be non-susceptible as before, race *6a* much less susceptible than it had been during the month of December, 1919, whereas *5a* conjugated more readily than at any other time during its history. Experiments with salt solutions were found to give essentially negative results. Three experimental cultures of each race were treated respectively with NaNO_3 (0.001 N), AlCl_3 (0.00003 N), and FeCl_3 (0.00002 N) on March 23rd, and another set was treated similarly on March 26th. The treated cultures of *17aIII* gave no conjugants, and those of *6a* and *5a* no more than could be found in the renewed cultures themselves.

Parallel experiments were likewise conducted with other strains of *Par. caudatum*. In the following series of experiments seven races (other than the ones considered above) were employed (table 3). Each had been cultivated under like conditions for one month, during which time conjugation occurred in culture *2a* on November 8th (5 per cent), but not in any of the other cultures. On November 14th, 1919, a dormant culture was started from each of these continuous cultures by diluting 100 cc. of culture with an equal part of distilled water, a few pieces

of solid hay being added to each to prevent starvation of the infusoria. These seven dormant cultures were renewed with hay infusion and distilled water on March 3, 1920, changed on March 6th and March 8th. On March 9th conjugants were found in one of these renewed cultures, *2a*, to the extent of about 5 per cent; on March 12th in culture *44a*, 5 to 10 per cent; and on March 17th in culture *57a*, about 5 per cent. The animals in *44a* continued to conjugate in increasing numbers after the 12th, reaching a maximum of 30 to 50 per cent on the 15th. A similar, but less extensive, epidemic manifested itself in *57a* during a period of many days, attaining a maximum of about 10 per cent. Experiments with various salt solutions were started on March 9th, and repeated on March 12th. In the first set of experiments, in which FeCl_3 and AlCl_3 were used in the optimal concentrations recommended by Zweibaum, negative results were obtained (March 10th). In the second attempt, AlCl_3 and NaNO_3 were used, the first one in two concentrations, and this time also essentially negative results were obtained, for although conjugation was already in progress in the culture *44a*, the relative number of conjugants in the experimental cultures was not appreciably altered as a result of the treatment with salts.

If these seven cultures be compared with the continuous cultures of the same races from which they were derived originally, it will be seen that the conditions in one set tend to parallel those in the other; that is, conjugation tends to occur in two distinct cultures of the same race even after they have been subjected to quite diverse conditions. For in the continuous culture *44a* (after the dormant culture had been derived from it on November 14th) conjugation took the form of an extensive epidemic between November 16th and December 15th, and in the continuous culture *2a* conjugation occurred on November 8th (5 per cent), and again, in small numbers, on January 11, and 12, 1920. No conjugation was observed to occur in the continuous culture *57a*, but in a subculture of this race, which had been set aside on October 31st with abundant nutriment, and allowed to undergo evaporation (method of Hance, '17),

conjugation occurred to the extent of 10 to 20 per cent on November 7th, after about one-fourth of the culture liquid had evaporated. Another observation indicates that this race was very nearly as susceptible as *44a* when the conditions were not maintained uniform. Four lines of this race which had been cultivated as isolation cultures on depression slides from October, 1919, until February 6, 1920, after being merged to form a mass culture, gave conjugation on the seventh day (February 13th).

Further consideration of the development of these races from the time of their isolation is instructive, for in two of them there was observed a periodic recurrence of conjugation under more or less uniform conditions of growth. In one race, *38a*, started as an exconjugant on March 25, 1919, it occurred on May 5th (10 per cent), and in the second 'generation' (i.e., the exconjugant race *38b*, started May 5th) on July 14th, in smaller numbers (1 to 5 per cent). No subsequent epidemic of conjugation has been observed in these cultures. The two races *28a* and *39a* were started on the same day as *38a*, each from an exconjugant, and were grown under like conditions; but in neither of these races has conjugation been observed during the first four months when the cultures were regularly examined, nor subsequently when examined only at intervals. Negative results were obtained with race *39a*, as with *38b*, in the experiments referred to above.

In the other 'periodic' race referred to (*44a, b, c, and d*), conjugation has been repeated four times. The original culture *44a* was started from an exconjugant on September 25, 1919, and gave rise to an extensive epidemic on November 16th, which lasted until December 15th. This involved practically all individuals in the culture, for as many as 5 to 10 per cent of the paramecia were in union at any one time during most of this period. In three out of four cultures of *44b*, started from corresponding exconjugants of *44a* on November 20, 1919, a large epidemic occurred as before, between the dates January 30 and February 23, 1920. Several new cultures, started on February 2 from exconjugants of *44b*, were collectively designated as *44c*, and in two of these likewise conjugation occurred, making

its appearance on February 28th in one, on March 2nd in the other. It made its first appearance in several exconjugant strains of the fourth 'generation' (44*d*, isolated on February 28th from a culture of 44*c*) as early as the 23rd of March. In the original culture, 44*a*, after the occurrence of conjugation during November and December, no more conjugants were observed until March 12, 1920, when they were found to the extent of about 5 per cent. Casual pairing occurred subsequently during a period of several days.

It is important to compare the reactions of these animals of race 44 with those of race 38 during their respective periods of conjugation. In the latter strain a slight disturbance in culture conditions (addition of hay infusion) caused immediate cessation of the process, whereas in the former it exercised but a temporary and partial influence when several times repeated. We have, as it would appear, two strains in which conjugation tends to manifest itself periodically, in one of which it is largely influenced through the environment, in the other more nearly independent.

The two races 43*a* and 57*a* were derived as exconjugants from the same source as 44*a*, on the same day (September 25, 1919). They were cultivated under parallel conditions and examined regularly during a period of six months, but no conjugants were observed in either of these continuous cultures grown under the same conditions as 44*a*. During the course of the experiments described above conjugation occurred in the renewed culture of 57*a*, but not in as great numbers as in 44*a*, whereas no conjugation took place in 43*a* after renewal.

In the remaining race, 8*a*, used in these experiments, conjugation has never been observed, either in the experiments or in a continuous culture maintained under the same uniform conditions as the continuous cultures of the six other strains (listed in table 3).

It is apparent from the results of these observations and experiments with several strains of *Paramecium caudatum* that no general directions may be formulated by which we can bring about conjugation in a given strain at any one time. When

various strains are compared under the same set of conditions they respond differently: in some conjugation occurs, in others it does not. Only one of the strains (*17a*) used in my experiments may be regarded as being similar to the strain which Zweibaum employed in his studies, and it responded to experimentation only during the first five months of its history. Other strains were found to conjugate readily in cultures which were renewed with hay infusion, after they had undergone a period of dormancy, but the use of salt solutions was not found to cause any appreciable increase in the relative number of conjugants, although these same solutions had been found effective in augmenting the intensity of conjugation in *17a*.

Others, moreover, persistently failed to give conjugants under uniform and under experimental conditions. Of these, certain ones (*39a*, *43a*) were derived originally from exconjugants, and so might perhaps be regarded as conjugating strains; but the race *8a* has never been seen to conjugate, although it has been under regular observation only during a period of six months. It is possible that these strains may in time become more susceptible to conjugation. Such a condition is indicated in the two races *5a* and *6a*, in which, after a period of about eight to ten months of cultivation, conjugation was found to occur in subcultures renewed after dormancy. Previous to this time cultures had responded negatively to the same treatment.

Taking the evidence as it stands, we would be forced to conclude that some at least of the strains studied here are heritably diverse. Tested by the experimental methods of Zweibaum, which he claims would be effective for any given race of *P. caudatum*, I have found consistent diversities between races as regards conjugation. My observations upon 'periodic' races, moreover, support this same general conclusion. It would be possible to point out other differences between the various races which I have studied, e.g., as regards size, shape, division-rate, etc., but since we are concerned here mainly with differences in respect to conjugation, I shall not attempt any such comparisons. Differences in respect to size and fission-rate have been studied in other races by Jennings and Hargitt ('10), but accord-

ing to Zweibaum these supposed racial diversities result from differences in the amount of nutriment. Supposing, however, that acquired diversities may arise in different cultures as a result of differences in environment, these same cultures should, in Zweibaum's opinion, yield conjugants after being subjected to the prolonged experimental procedure which he recommends. But this they fail to do in all cases, as the results of my experiments indicate. Cultures which are known to have a common ancestry (various cultures of the same pedigreed stock, descended from one original individual) do respond similarly in these experiments. But when races of diverse ancestry are compared they almost invariably respond differently. Two races coming from the same habitat (as from the same pond or stream, aquarium, or hay culture) may sometimes respond similarly under the same conditions, but as a rule they respond differently. Thus, the two races 38 (*a* and *b*) and 39*a* were from the same general source, a pond near Baltimore, but the first underwent two epidemics of conjugation in the laboratory, during which time no conjugation has been observed in 39*a*. Similarly, the two races 44*a* and 43*a* were isolated from the same lot of material collected in a river. In 44*a* (and its derived cultures) conjugation has occurred in epidemics of long duration under essentially uniform conditions, whereas in the latter strain (43*a*) no conjugation has been observed since the beginning, under uniform conditions or under the altered conditions of growth which favored conjugation in 44*a* (as when cultures are renewed after dormancy).

Summary of observations and experiments on Paramecium caudatum. 1. There are diversities among the various strains of this species as regards their tendency to conjugate under natural and experimental conditions.

2. Some of these diversities in response under a given set of conditions may be qualitative, as illustrated by the different behavior of two conjugating strains when treated with a particular reagent. That is, the conditions for conjugation in all strains are not identical.

3. That the several strains in question are racially diverse is supported by the fact that conjugating strains sometimes

maintain their ability to conjugate freely for several 'generations,' in new cultures started from exconjugants of a previous epidemic.

b. Paramecium aurelia

My studies upon races of this species open up problems essentially like those presented by *caudatum*. Some of the cultures were subjected to daily observation during the first weeks of their development. Conjugation was thus shown to be of frequent periodic occurrence in some, rare and apparently sporadic in others, while in some it was found neither during this period of daily observation nor on subsequent examination at intervals of two to five days for several months. At intervals dense watch-glass cultures, derived from the main cultures, were set aside in moist chambers and examined the following day for conjugating pairs. In such cultures the nutriment is quickly used up by the paramecia, and the conditions are right for an epidemic of conjugation, provided the organisms were taken from a flourishing culture (method of Maupas, '89). In this manner some of the races which showed no conjugants in the stock cultures were made to give several in these isolation experiments, while others, which showed small numbers in the stock cultures, yielded as many as 50 per cent in the experiments. As a rule, pairing took place earlier and persisted later during a series of such experiments than in the main cultures themselves, when the experiments were begun sufficiently in advance of an epidemic of conjugation in the main culture. That is, conjugation could generally be obtained in the dense watch-glass cultures several days before any pairs were seen in the stock culture from which they had been derived, and could be obtained for days after it had ceased in the stock culture. The effect in such experiments is, then to augment conjugation in cultures of organisms already in a state of susceptibility. Certain races, however, failed to conjugate during a period of several months, even when placed under the more favorable conditions mentioned above (isolation experiments). In this connection, also, solutions of various salts were added to some of the watch-glass cultures when isolated. This served to increase somewhat

the percentage of the conjugants in certain more susceptible races, but many that did not conjugate still remained.

From the above it will be seen that among the various strains of *Paramecium aurelia* there appear to be several intergrading conditions as regards conjugation; in some races it occurs spontaneously, in others its occurrence is conditioned (in the laboratory) by certain experimental proceedings—alterations in environmental conditions. The question arises whether we cannot by still other, perhaps more indirect, methods bring about conjugation in all races of this species. So far as the results of my work go, the question may be answered in the affirmative. Of the thirteen races of *P. aurelia* studied in the beginning, all proved amenable to indirect methods. Six were found to be conjugating races at one time during their existence; that is, they conjugated under more or less uniform conditions of cultivation. Seven were found to be more refractory, yielding only to indirect methods.

The most effective procedure for these particular races was to subject them to a period of semistarvation lasting from four to eight weeks, then to bring each to a state of rapid multiplication by adding hay infusion containing certain electrolytes to the culture. The culture medium used most effectively contained NaNO_3 , final concentration about 0.001 to 0.004 N, and a smaller proportion of $\text{Ca}(\text{NO}_3)_2$, (0.0001 to 0.0004 N). The solution used in later experiments contained NaNO_3 alone, and this gave about as good results. It was thought that the calcium used in these preliminary experiments would offset the toxic effect of sodium, and so lead to better growth in the culture, but it was found that the sodium salt when used in low concentrations (0.001 to 0.003 N) was relatively non-toxic to most races of *P. aurelia*. As will be shown later, the effect of sodium salt upon *Paramecium* is to stimulate growth and thereby accelerate the fission-rate.

The period of semistarvation to which it was found necessary to subject each culture differed considerably, depending upon the race employed. Some few of these (14a, table 5, and 16a) proved to be very refractory, for they failed to give conjugants

except after fasting for eight to nine weeks. Others, on the other hand (e. g., *13a*, table 4, and *25a*), conjugated after shorter periods of dormancy—about three to four weeks. The duration of this period serves perhaps as the best criterion for judging the relative susceptibility of various races toward conjugation, for experiments with salt solutions generally give negative results unless the organisms have become susceptible to conjugation previously through dormancy.

The effects produced by using salt solutions directly in experiments designed to induce conjugation (method of Zweibaum) are rather variable and unpredictable. As a rule, the salt solutions increase the percentage of conjugating pairs, but I suspect that their action is largely osmotic rather than chemical. When added to the main cultures when renewed, however, the effect of sodium salt is more marked, although even by this method a relatively small number of conjugants is obtained. As examples of this effect may be cited experiments with race *13a*, table 4; *14a*, table 5; *11i* and *11j*, table 6, and *3d*, table 7. The reactions of these various races, however, may be contrasted with that of race *1b*, table 7, in which conjugation is not favored by the presence of sodium nitrate, or, if so, only by a lower concentration than that which was found most effective in the other races.

In order to bring out more clearly some of the racial differences between the various strains of *P. aurelia*, the following observations are recorded. They indicate, also, something regarding the nature of the 'periodicities' in conjugation so characteristic of many strains of this species.

Par. aurelia, 1 (*b* and *c*). This race was descended from a single individual isolated January 30, 1919, from an old laboratory hay culture. The original culture, *1b*, developed very rapidly under laboratory conditions, the animals proving to be quite hardy at all times during their history.

A watch-glass culture containing numerous individuals was set aside on March 17th, and on the following day showed conjugation involving about 10 per cent of all. Observations failed to reveal the occurrence of the process in the stock culture

previous to the 19th, when one or two pairs were noted. It was recorded at intervals during March and April, but on each occasion in very small numbers (involving usually less than 1 per cent). During a period of thirty-two days (March 17th until April 18th) very frequent recourse was had to isolation experiments, in watch-glass cultures, in order to determine whether the organisms continued susceptible. Every one of these experiments yielded conjugants, in numbers ranging from about 5 per cent to as high as 50 or 60 per cent in some instances. Throughout most of this period no conjugation was noted in the stock culture, either on the day preceding each isolation experiment or on the same day. It frequently happened that in a dense culture set aside in a watch-glass conjugation set in almost immediately, so that at the end of one-half hour 10 per cent of the paramecia would be found in union, although no pairs had been seen in the beginning. Other races responded similarly in these experiments, but not so quickly nor in such large numbers.

The strain *1b* was subjected to experimentation on September 30, 1919, when renewed cultures were started in small Stender dishes with material taken from the stock culture. This had been allowed to stand for over three months without renewal, although considerable hay was added to it previously. The animals were found to be in a very healthy state, showing no effects of starvation, although they were not dividing. Practically no conjugants had been observed in the culture since May, 1919, when casually examined. On the second day after renewal conjugation was obtained (on October 2nd) in the culture *1b-A* to which no electrolytes had been added. In the other cultures, containing NaNO_3 , pairing took place in smaller numbers (table 7).

As was pointed out above (p. 358), the reaction of these organisms to the presence of NaNO_3 in the renewed culture is in marked contrast with that of the other races to be described later, for which a low concentration of this salt acts as a stimulant to conjugation. The animals of culture *1b* (and of *1c*) always conjugated most readily in an aqueous medium, whenever

they conjugated at all. In the stock cultures the paramecia conjugated little or not at all, but they remained susceptible during long periods of time, yielding conjugants readily in watch-glass cultures isolated from the main cultures.

Par. aurelia, *3a*. The culture was started January 23, 1919, from an individual isolated from the same abandoned hay culture as *1b*, and so it is not known with certainty whether it represents a race distinct in descent from *1b*, although its characteristics are sufficiently distinct to render this probable. Conjugation was observed in this culture while still small on February 13th, in about 20 to 30 per cent of all, and again on February 15th and 17th in smaller numbers (3 to 5 per cent). Conjugants were again seen in this culture on March 12th, about 1 per cent, and later between March 17th and 22nd, in no case in more than casual numbers. Isolation experiments continued to yield them, however, often as many as 5 per cent, from March 15th until April 18th, when the experiments were discontinued.

Culture *3b* was derived from an exconjugant of *3a* isolated on February 13th. One or two pairs of conjugants were seen in the culture on April 7th, after long search, while isolation experiments yielded them in small numbers (1 per cent or less) between March 19th and April 10th. Except for the casual pairs noted above (April 7th), the stock culture showed none from its beginning until the time when regular observations were discontinued.

Cultures *3c*, *3d*, and *3e* were started on March 20th from three exconjugants of *3b*, and represent thus the third 'sexual generation' of the race. Each was cultivated under uniform conditions and observed regularly. Conjugation occurred to the extent of 40 per cent in a watch-glass culture derived from *3d* on April 16th, but was not discovered in the main culture during the entire period of daily observation (from March 20th to April 20th), nor on subsequent examination at longer intervals. Isolation experiments set before and after the 16th (on April 15th, 17th, and 18th) gave no conjugants. The two related cultures, *3c* and *3e*, failed to show any conjugation, even in the isolation experiments, which were continued until April 20th.

Comparing this race, no. 3, with race 1, it will be seen that in both conjugation occurred naturally under the more or less uniform conditions afforded in large stock cultures. In culture 3*a* there is at first apparently a periodicity marked by 'epidemics' in the occurrence of the process at intervals of about four weeks. In culture 3*b*, started from an exconjugant, the first epidemic appeared after about five weeks, and in 3*d*, similarly related to 3*b*, after about four weeks. Conjugation in race 1*b*, while not occurring at periodic intervals in the stock culture, was readily evoked in isolation experiments during the entire period when such experiments were regularly conducted (March 18th to April 18th). The same irregularity tends to manifest itself in the culture 3*a* after the onset of the second period of conjugation (March 12th).

This irregularity in the occurrence of conjugation during the later stages of growth in a culture in which it has already taken place, in the form of a preceding 'epidemic,' makes it impossible to measure accurately the interval between conjugation periods except with cultures started from exconjugants, and in which conjugation has not previously occurred. Even the first epidemic of conjugation in a newly developed strain may show two separate phases (see observations on races 11*c* and 11*f*, p. 363) suggesting that diverse lines, showing acquired differences in susceptibility as regards conjugation may have arisen among the offspring of one individual. Later irregularities in conjugation—after a previous epidemic in the same culture—may perhaps be accounted for in part, also, as due to hereditary differences established through conjugation, among the exconjugants of the first epidemic. Thus diverse strains, with different degrees of susceptibility, each having perhaps a somewhat different periodicity, might be perpetuated in the same culture coming originally from the same race. If this be true, a new clone derived from a single exconjugant would presumably give conjugants in a more sharply defined epidemic than a culture containing the offspring of several exconjugants, although originally from the same strain.

Some such interpretation as this may account for the irregular occurrence of the process in the later history of the mass culture *3a*, for a study of the later generations (*3b* and *3d*) shows that conjugation in these, like the first epidemic in *3a*, is sharply defined. Although no sufficient evidence is here afforded that hereditary differences exist between the corresponding cultures of the same generation of exconjugants (*3c*, *3d*, and *3e*) in respect to conjugation, some observations made upon exconjugant lines of the race *44* (*P. caudatum*) indicate that such heritable diversities do arise in this manner (p. 373).

P. aurelia, *11a*, was obtained as a mass culture on February 15, 1919, from Professor Jennings, by whom the race had been reared from one individual isolated January 18, 1919. Conjugation had already been observed in this culture on February 5th, and again on February 15th, involving at each epidemic a fair percentage of individuals. On March 5th and 8th pairing was observed in about 10 per cent of all, and from March 10th to 12th its occurrence was noted as casual. Conjugants were seen again, in smaller numbers (about 3 per cent), on April 4th, although casual pairs were found as early as March 29th, and later, on April 9th. The process, it will be seen, is becoming more irregular in occurrence as time progresses, the separate epidemics becoming much less distinct and the percentage obtained at any one time falling off. This is evidenced, also, by the fact that, although conjugants were not discovered in the main culture between March 12th and 29th, yet isolation experiments continued to yield them in considerable numbers (5 to 30 per cent) between these dates. Three, and possibly four, successive epidemics are thus indicated for race *11a*. Results of later observations (in races *11c* and *11f*, p. 363) showed that the occurrence of two sharp epidemics close together in point of time, such as those of February 5th and February 15th, or March 5th and March 8th, may very properly be regarded as one, the first onset being interrupted by an abrupt change in nutritive conditions (the addition of hay infusion to the culture, etc.).

Cultures *11b*, *11c*, *11d*, and *11f*. These were derived from corresponding exconjugants of *11a* isolated on March 20th. A well-marked epidemic of conjugation, involving about three-fourths of the culture, occurred in *11c* on April 4th. The process was repeated on April 14th (about 5 per cent), and continued until April 18th (casual). In culture *11f* conjugation appeared first on April 15th (casual), and on the day following, to the extent of about 60 per cent. On April 17th about 10 per cent were in union. No conjugants were observed in *11b* and *11d*, examined daily until April 18th, and casually thereafter.

Exconjugant strains of *11c* were started from ten pairs isolated April 4th, and of *11f*, from six pairs, on April 17th. The twenty exconjugant cultures of *11c*, designated as races *11g* (1 to 20), were grown under very uniform conditions, in hay infusion made up with distilled water, and renewed each week. Under the favorable conditions thus imposed, no conjugation was observed in any of the cultures for over two months. Similar negative results were obtained with the twelve strains derived from *11f* exconjugants, designated as *11h* (1 to 12), subjected to daily observation for six weeks and examined casually afterward.

On the 6th of July an individual of *11g* (from a culture which had lain dormant for about a month) was isolated in a watch-glass, in hay infusion containing 0.003 N NaNO_3 . On July 10th conjugation occurred among the numerous progeny to the extent of 15 per cent. From the exconjugants two daughter strains were started (*11i* and *11j*). No conjugation was noted in these cultures prior to July 24th, when they were left unobserved and unaltered.

In each of these conjugation was again obtained in small numbers during September by renewing the dormant cultures with hay infusion containing NaNO_3 (table 6). No conjugation occurred, however, in those cultures which were renewed with hay infusion alone.

The study of *11a* and its descendants reveals a condition which is prevalent in various other races of this species (e.g., *3a* and *23a*), as well as in *caudatum*. Conjugation was found to occur very readily in cultures of the race during the first

few months of its history, usually under fairly uniform conditions; whereas later the process could be evoked only after renewing cultures which had undergone dormancy. The natural periodicity which characterized the early stages became suppressed, and the relative number of conjugants obtained likewise fell off considerably as time progressed. The effect is probably not due to unfavorable culture conditions long maintained, although this may play some part, nor to close inbreeding; but is probably connected in some way with the adjustment of the organisms to a state of continuous, rapid multiplication. This point will be treated more at length in part 2 of this paper (p. 371).

The eight races designated *13a*, *14a*, *15a*, *16a*, and *23a*, *24a*, *25a*, and *26a* were derived on February 28, 1919, from the same source—an abandoned hay culture containing various infusoria. Each was descended from a single exconjugant, the original individuals of *13a* and *23a* having come from the two members of one pair, *14a* and *24a* from another pair, etc. It is important to compare these races from the start, since all had been subjected to the same environmental influences previously. The cultures were examined frequently for eight weeks, while numerous tests for conjugation were made with dense watch-glass cultures isolated from the main jars. All the stock cultures developed rapidly, except *24a* and *26a*, in which growth was more tardy, owing in part to overnutrition, to some extent possibly to racial weaknesses. Conjugation occurred in two cultures, *23a* and *25a*, during the period of continuous cultivation, but not in the six others.

In *23a* several pairs were observed as early as March 11th, eleven days after the previous union, and again on March 17th. They were found in larger numbers (5 to 15 per cent) from March 18th to 22nd, but only casually thereafter until March 31st, when conjugation practically ceased in the continuous culture. Isolation experiments gave as high as 40 per cent on March 15th, and subsequently until April, in diminishing numbers (March 18th, 40 per cent; March 20th, 30 per cent; March 26th, 5 per cent, etc.).

The epidemic in *25a* was less intense, involving at any one time a much smaller percentage of organisms, and lasting but a short time (Mar. 18th to 25th). Isolation experiments yielded conjugants during a somewhat longer period, but in scarcely any greater numbers.

The sequence of events in these two cultures is seen to be very similar to that which obtains in such races as *3a* and *b*, *11a* and its derivatives. That is, there is a period of vegetative growth lasting from two to three weeks, after which conjugation ensues. The duration of this period of conjugation depends in large measure upon the susceptibility of the races in question, beginning earlier and lasting longer in the more susceptible ones—except in some few instances where, in small cultures, the process is consummated in one or two days (e.g., *11f*).

In order to determine whether these races (*23a* and *25a*) which had conjugated under uniform conditions had really done so as a result of their greater natural susceptibility, and not, as might be supposed, because of more favorable environmental conditions, new strains were started from isolated exconjugants of each. In this way the initial medium was greatly altered, new strains of bacteria coming in, with the elimination perhaps of many strains of bacteria present in the old cultures. Of the two strains derived from exconjugants of *23a* which were reared successfully, conjugation occurred in each, approximately three weeks later. In one of these, *23f*, isolated March 21st, conjugants were observed on April 14th (5 to 10 per cent), April 15th and 16th (casual). In the other culture, *23i*, isolated March 21st, conjugation was only casual, one pair being seen on April 12th in a mixed culture of two races, *P. caudatum 17a* and *P. aurelia 23i* (table 2). Two cultures of *25a* exconjugants (*25e* and *25h*) started on the same day as those from *23a*, and similarly treated, showed no conjugants, even in isolation experiments. The relative susceptibility of these two races, of the second generation, to each other is thus shown to be the same as for the two parental strains. That is, the race *23* seems more susceptible than *25* in each case, although conjugation failed to

occur in the second generation of 25, and was less intense during the second epidemic (second generation) of 23 than during the first.

In the six remaining stock cultures (of races 13a, 14a, etc.) no conjugation was found to occur during the first five months while continuous cultivation was resorted to, although it was obtained by indirect (experimental) methods. Those experiments in which salt solutions were used in the way recommended by Zweibaum gave less striking results in this species than in the one caudatum race, 17a, in which they were effective. Compare, for example, the results of such experiments with race 13a (*P. aurelia*) with those in race 17a (*P. caudatum*) when both strains were growing in the same culture (table 1). As was pointed out on page 358, cultures of nearly all races, when renewed with hay infusion containing NaNO_3 (0.001–0.004 N) may give conjugants when none can be found in controls, renewed with an aqueous medium.

The duration of dormancy preceding the growth period is perhaps the most potent influence favoring conjugation, for it was only by renewing cultures which had long been dormant that conjugation could be elicited at all in such races as 14a (table 5), and 16a. In these two strains a preliminary period of dormancy of seven to eight weeks was required before conjugation could be obtained in the cultures renewed with a saline medium. With other races, e.g., 24a and 25a, only three to four weeks of dormancy were required before renewal in the same way. One of these (25a), it will be noted, had been found earlier to conjugate under uniform conditions (in the stock culture), although it had later lost this tendency. It was shown afterward that three months of dormancy was sufficient for conjugation in any of the races of *aurelia* when renewed with an aqueous medium (tap-water), for when the stock cultures were renewed in October, 1919, after this period of dormancy, a few conjugants were seen in all of those cultures in which none had been discovered previously, as well as those in which they had been found before.

Summary of observations and experiments on Paramecium aurelia. 1. In this species, as in the preceding, there are diversities in respect to conjugation. Some strains show a marked tendency to conjugate repeatedly at regular intervals, while others, under the same uniform conditions, do not conjugate during long periods of time. Diversities in response to the same set of experimental conditions are sometimes indicated, as when one race shows a greater tendency to conjugate in a particular saline medium than in an aqueous medium, another race so treated showing a lesser tendency.

2. These diversities probably have a racial basis, for if two cultures of the same strain, each having had a similar history, be subjected to the same treatment they show about an equal tendency to conjugate. Furthermore, when two different strains are compared they are found to preserve about the same relative degree of susceptibility, under the given set of conditions, as they showed in the beginning.

Comparing, now, these two species of *Paramecium* as regards conjugation, we find certain points of resemblance as well as differences. They each respond, in general, to the same set of experimental conditions—dormancy, renewed division, etc.—and show some similarity in their behavior when treated with salts. In each species there is a tendency for conjugation to occur periodically in some strains, although the intervals between periods are not of like duration. In the smaller species (*P. aurelia*) the life processes appear to be contracted into a shorter space of time than in the larger, but the same kind of internal factors seem to be operative in both species.

The rôle which these internal factors play is best shown when we subject a strain to a uniform environment, or by testing experimentally (using the same experiment each time) at frequent intervals during a period of several months. Studied in this way, the organisms show periods of greatest susceptibility and refractory intervals during which conjugation does not occur.

On the other hand, there is reason for believing that the internal factors may be slowly modified with time, perhaps by the prolonged adjustment of the organisms to a new environment. Strains which early in their history showed a marked tendency to conjugate periodically lose this power as time progresses. Evidence bearing on this point has been presented: for *Par. caudatum*, race 17*a* on page 349; race 38, page 352, and for *Par. aurelia*, race 3, page 360; race 11, page 362, races 23 and 25, page 365. Those races which, early in their history, showed a tendency to conjugate at frequent intervals, and in large numbers at each epidemic, retain their susceptibility longer than those races which at first conjugated only at longer intervals and in smaller numbers. The susceptibility of such cultures in which conjugation is in abeyance can often be partially restored by subjecting them to a long period of dormancy. Thus, a culture of *Par. aurelia*, 23*i*, renewed on January 10, 1920, after a five-month period of dormancy, gave conjugants on January 16th. From an exconjugant a new culture, 23*k*, was started, and in this conjugation was repeated under essentially uniform conditions. (In several new exconjugant lines, 23*l*, (1) to (6), however, conjugation did not occur.) This shows that the very long dormant period to which the organisms of this race were exposed had restored their power to conjugate periodically, as in the original culture, 23*a*, of this race (p. 364).

2. GROWTH FACTORS INFLUENCING CONJUGATION

On the basis of the above observations, and by the aid of supplementary experiments, I shall attempt to analyze the conditions which are known to influence the occurrence of conjugation in *Paramecium*. Three outstanding factors or relationships call for explanation. These are, 1) the relation of fission-rate to the onset of conjugation; 2) the state of dormancy, and, 3) the periodicity which characterizes freely conjugating strains.

A comparative study of division-rate as such, in the various races of *Paramecium* here treated, shows that there is no simple correlation between high or low division-rate and a strong tendency to conjugate. Among those races having a high rate of

fission, and resembling each other closely in size and appearance, there are those which conjugate repeatedly and those which do not, and the same may be said for the slowly dividing strains.

But it seems probable, in the light of our experimental knowledge, that there is an indirect connection between conjugation and fission-rate. Whenever conjugation occurs in a culture en masse, it usually does so after a period of accelerated division. This relation has been emphasized by several investigators, working with diverse genera of Infusoria, both free living and parasitic. In fact, Maupas regarded it as one of the primary conditions preceding a conjugation epidemic in the many species which he studied, and applied it practically in his work.

In further support of this view—that conjugation is conditioned by a preliminary period of increased division—we may recall the effects produced by adding salts to the cultures of *Par. aurelia* when renewed. In a medium of sodium nitrate (of 0.001 to 0.005M concentration) conjugation is almost invariably augmented in this species (cf., however, race *1b* to the contrary, which conjugated as well in an aqueous medium). Oftentimes conjugation can be elicited in such 'salt cultures' when none occurs in the controls renewed with an aqueous medium. It may be shown very easily by means of slide cultures, changed daily, that NaNO_3 in such concentrations acts as a stimulant to growth. The division rate in these treated lines may be maintained at a considerably higher level than that of the controls for at least two weeks (table 8). Some races apparently do not tolerate the continuous action of the salt and die after two or three days of accelerated growth, and the death rate is greater in any race so treated than in the controls. These observations apply to races of both species of *Paramecium*, but only occasionally is a race of *caudatum* found which will tolerate the action of the salt as used in these experiments.

J. Spek ('19) has conducted similar experiments on *Par. caudatum*, using various salts in the medium, and finds that many of these tend to accelerate fission-rate.

My experiments upon *Paramecium* using MgSO_4 in the culture medium indicate that this salt has a depressing effect upon

growth. Most races become readily adapted to a 0.01M concentration, and may continue to live for months in such cultures to which hay has been added. The division rate is not appreciably diminished by this salt, although extensive experiments may yield more definite results than I have thus far attained. In the few conjugation experiments in which MgSO_4 was used (*P. aurelia*) the results were negative, except when the solution was used indirectly to increase the osmotic concentration of the medium in a derived culture. These cultures, when renewed with such a medium, continued to live, but failed to grow with sufficient rapidity to develop the initial impulse to conjugate (ex hypothesi).

Let us now consider the second condition, or antecedent, to conjugation, namely, that of subjecting the cultures to a period of dormancy. From what we know concerning the relation of fission-rate to conjugation, it might be conjectured that such a period of rest (dormancy) renders the organisms so treated potentially more capable of rapid division than those which had been dividing previously. Or, conversely stated, a regulation in division-rate may occur in organisms subjected to long-continued multiplication, so that they do not divide so fast as formerly. Such a regulation has been shown by Jollos ('13) to occur in paramecia subjected to a higher temperature. Lines which had been growing at 19°, after being transferred to 31°, divided at a rate of 9 per forty-eight-hour period; after six months at this temperature, however, at the rate of 7.

If any regulation in division-rate does occur in organisms subjected to nutritive conditions favoring rapid growth, one ought to be able to test it experimentally, using two cultures of the same strain, one of which had lain dormant for several months, the other having undergone continuous growth. Accordingly, such an experiment was tried. A race of organisms (*P. caudatum* 39) in which conjugation had never been observed was chosen in order to eliminate any secondary effects which might accompany this process. The culture 39A had been maintained under rather uniform conditions of growth, renewed with hay infusion every two or three weeks for a period of about six months,

during which period the culture 39a had lain dormant. Both cultures were examined daily during the two weeks preceding the experiment, but showed no conjugants. The mean division-rate in fifty-two lines from 39A was found to be $11.25 \pm .175$ for a twelve-day period; the mean rate in forty-four lines from 39a was $15.70 \pm .193$ for the same twelve-day period. The effect of long-deferred division, i.e., dormancy, is, then, to render the organisms capable of more rapid division when favorable growth conditions are afforded. It follows from these results that dormancy may influence conjugation through its indirect effect on fission-rate, if it be true that rapid division induces conjugation. This may serve to explain, also, why it is that conjugation occurs most readily in cultures of infusoria which have but recently been brought into the laboratory and subjected to conditions producing rapid growth. For obviously the conditions in ponds and streams are not ordinarily such as to maintain these animals in constant division, as we find in a fermenting hay culture, so that when brought into the laboratory they are in essentially a dormant condition, and will multiply rapidly when the conditions are right. On the other hand, the tendency for conjugation to become suppressed in previously conjugating strains, after prolonged cultivation (p. 368), receives an explanation: it is a phenomenon accompanying the gradual loss of the power of division.

A third line of evidence favoring the view that conjugation is initiated by a period of unregulated division is obtained from the records of fission-rate in strains which conjugate at regular intervals. As pointed out by Hertwig ('89) and fully demonstrated by Jennings ('13), the most apparent immediate effect, produced by conjugation is a decrease in fission-rate. This lowering tends to disappear completely in about two months (*P. caudatum*). My observations on conjugating strains indicate a similar condition which may be repeated after each recurrence of the process; and this has led me to think that the periodicity in conjugation may in some way be controlled by this tendency for fission-rate to increase gradually to a high point. This is the essential idea involved in Hertwig's explanation of conjuga-

tion ('89) as a process following a period of unregulated division, although he recognized also an alternative process of nuclear reorganization ('parthenogenesis') in *Par. aurelia*. He says (p. 226):

Für das, was ich beweisen möchte, genügen die Resultate in ihrer jetzigen aphoristischen Form; sie zeigen, dass fortgesetzte Theilungen im Infusor eine Neigung zur Conjugation veranlassen, dass aber bei künstlicher Verhinderung der Vereinigung die Theilungen in lebhaftester Weise fortgesetzt werden, bis ein Moment eintritt, in welchem ohne Conjugation ein Ersatz des Hauptkerns durch Abkömmlinge des Nebenkerns eintritt.

Wer die Conjugation der Infusorien erklären will, muss dem Gesagten zufolge mit 2 Erscheinungen rechnen: 1. fortgesetzte Theilungen ohne Conjugation führen zum Untergang; 2. trotzdem besitzt das Infusor zur Zeit der Conjugation unverminderte oder sogar erhöhte Theilfähigkeit. Wenn wir Bütschli's Theorie zu Grunde legen, stehen beide Sätze miteinander im Widerspruche; sie sind aber sofort in Einklang zu bringen, wenn wir annehmen, dass zur Zeit, wo die Conjugation eintritt, nicht eine herabgesetzte, sondern eine übermässig erhöhte Lebensenergie besteht. Dann hat die Conjugation nicht den Zweck, die Lebensenergie noch weiter zu steigern, sondern die gesteigerte Lebensthätigkeit so zu regulieren, dass sie nicht zur Zerstörung des Organismus führt; sie heilt nicht die durch physiologische Usur entstandenen Defecte, sondern verhindert, dass derartige Defecte durch Uebermass der Function entstehen.

His later observations ('04) on *Dileptus* are likewise in accord with this earlier expressed view.

The following observations, which were made at various times during the course of my work, have a bearing upon this point. In the race *44a*, of *Par. caudatum*, started September 25, 1919, from an exconjugant, the fission-rate was found to attain to a maximum during the period from November 15th to November 28th (eighth and ninth weeks) in four lines started in October. And it was at this time, also, that conjugation was extensive in the stock culture of *44a*, commencing on November 16th and lasting until December 15th (eighth to eleventh weeks). From exconjugants of this culture sixteen new lines were started—*44b* (1) to (8), two lines from each. Both lines of *44b* (5) died after six weeks, but from the remaining strains, four mass cultures were derived: *44b* (2) and (3) on January 9th, *44b* (7), and (8) on January 21, 1920. Conjugation occurred extensively in

three of these mass cultures, during approximately the same period of time in each, beginning on January 30th in 44b (7) and lasting until February 27th. Just preceding conjugation, during the tenth week, the fission-rate in the slide cultures reached the highest point which it had attained since the second week following the previous conjugation, as shown by the average number of divisions weekly for all the lines. (This first maximum may possibly represent a compensatory tendency after the drop immediately following conjugation, for the average rate for the first two weeks is less than that for the second two weeks.) New strains were again started from exconjugants of the culture 44b (2) on February 2, 1920, and were designated 44c (1) to (12). From the six which survived, mass cultures were started on February 16th, and in two of these conjugation set in at the end of the fourth week. The fission-rate for these two strains attained its maximum during the fifth week. A similar set of experiments was conducted, using forty exconjugant strains derived from one of the above cultures, and designated 44d (1) to (40). Twenty mass cultures were started, and in six of these conjugation occurred, setting in during the fourth week in each one. The fission-rate (in four lines of each strain) attained its highest point at about the third week. It is significant in this connection to note that in almost every instance conjugation occurred in rapidly dividing strains. Slow strains, although derived originally from exconjugants of the same race, do not conjugate, even after a period of three months has elapsed.

These experiments bring out another point which has already been touched upon (p. 362). There appear to be racial diversities among the exconjugant strains, originally from the same race. When a particular strain shows a tendency to undergo depression, all of its four lines may be affected, and frequently all die at about the same time (usually at the end of four to six weeks), whereas in the four lines of some other strain no depression may occur at this time.

My experiments with *Par. aurelia* likewise support the view set forth above: that conjugation occurs at about the time

when division is at its maximum or shortly afterward. Thus, in the strains of *23k* (p. 368) the fission-rate was highest during the second week, and conjugation occurred (in one culture) during the third week.

All this lends support to the hypothesis that it is the unusual conditions of rapid growth which predispose to conjugation. Circumstances which favor rapid growth may likewise favor conjugation. Abundant nutriment and the presence of reagents which stimulate growth without harming the organisms act in this way, but for these conditions to operate most effectively the organisms themselves must have come from freely conjugating strains, rendered potentially more capable of rapid division through dormancy.

GENERAL SUMMARY

The following conclusions regarding the causes and periodicity of conjugation in *Paramecium* may be drawn from the observations and experiments above set forth.

1. Various strains of the same species display different degrees of susceptibility, or readiness, to conjugate when taken from a similar environment and grown under like conditions.

2. They may likewise show diverse reactions as regards conjugation when subjected to the same experimental conditions, as when treated with a certain salt solution.

3. A high degree of susceptibility is indicated by a tendency for conjugation to appear in successive 'epidemics' within a given strain and by the relative number of individuals involved.

4. The observed time interval between conjugation periods in *Paramecium caudatum* is from four to twelve weeks. Conjugation was recurrent in culture *38a* after six weeks, in *38b* after ten weeks. In culture *44a* it occurred after seven to eight weeks, and again after twelve weeks, but in the smaller cultures derived from it (*44b*, *c* and *d*) at intervals of about four to eight weeks.

5. The time interval in *Paramecium aurelia* is from two to six weeks (pp. 361 and 365).

6. There exist strains which do not conjugate during long periods of time, and in which, were the conditions maintained

uniform and favorable for growth, the process might probably be postponed indefinitely (cf. Woodruff's culture).

7. Many of these races which do not conjugate can be rendered susceptible to conjugation by subjecting them to a prolonged period of reduced nutrition, thus inhibiting multiplication. Such 'dormant' cultures, of any race, when renewed by supplying abundant nutriment, may yield conjugants more readily (if conjugation occurs at all) than control cultures of the same strain which have been kept under conditions of constant growth.

8. The addition of certain salt solutions to samples taken from these renewed cultures after a few days of growth may give an increase in the relative number of conjugants in certain races, without affecting appreciably the proportion of conjugants in other races of the same species (as worked out especially for *Par. caudatum*).

9. A small amount of sodium nitrate added to the renewed cultures at the time of their renewal, serves to augment conjugation in many races of *Par. aurelia*. Diverse races appear to react differently toward equal concentrations of the salt as used in this way, for race *1b* and its derivatives underwent conjugation more readily in aqueous medium than in the solutions of the salt in concentrations found most effective for the other races of this species. This same race multiplied more steadily and maintained its numbers longer in aqueous cultures than other races of this species.

10. There is a progressive tendency, probably in all races, for conjugation to become suppressed after prolonged cultivation under laboratory conditions (p. 368). The susceptibility of such cultures in which conjugation is in abeyance can often be partially restored by subjecting them to a long period of dormancy, as described under point 7.

11. This restored power of conjugation in organisms subjected to long dormancy is correlated with their higher rate of fission, as compared with those organisms of the same race which have previously been undergoing division.

12. From what is known regarding the effects of salts in accelerating fission and in augmenting conjugation, it appears that conjugation is initiated by a period of unregulated division.

13. The periodic occurrence of conjugation in certain strains of *Paramecium* may likewise be brought into relation with periodic changes in the division-rate of these strains.

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EXPLANATION OF TABLES

In the tables which follow (1 to 7, inclusive) are recorded the results of experiments designed to induce conjugation in the various races of *Paramecium* (*aurelia* and *caudatum*). The tables indicate how long each culture was subjected to dormancy, when renewed, and the nature of the culture medium. Distilled water (and hay infusion) was used except as otherwise noted. The concentrations of 'salt cultures' are expressed as final concentrations for the salts in question.

Conjugation is expressed, when absent as 'none;' when present, in terms of the percentage involved (approximate). 'Casual' conjugation is about 1 per cent or less, or an indefinite small number.

Unless otherwise stated, the experiments with salts (and other reagents), given in the last column of some of the tables, were conducted, using one part of culture containing numerous paramecia and three parts of the reagent.

TABLE 1

DORMANT CULTURE	DATE RENEWED; CULTURE MEDIUM	CONJUGATION IN RENEWED CULTURE	SUBCULTURES 24 HOURS AFTER TREATMENT: CONJUGATION
<i>P. caud. 5aII</i> , March 28, 1919	A. April 11 (aqueous)	None	April 18, untreated None
	B. April 23 (aqueous)	None	April 28, untreated None
			May 3, + NaNO ₃ (0.001N) None
			May 3, + FeCl ₃ (0.00002N) None
	C. May 9 (aqueous)	None	May 14, + NaNO ₃ (0.001N) None
			May 14, + FeCl ₃ (0.00002N) None
	D. May 28 (aqueous)	None	May 26, + NaNO ₃ (0.001N) None
			May 28, + NaNO ₃ (0.001N) None
<i>P. caud. 6aII</i> , March 28, 1919			May 26, + FeCl ₃ (0.00002N) None
			May 28, + FeCl ₃ (0.00002N) None
	A. April 11 (aqueous)	None	April 14, untreated None
			April 18, untreated None
	B. April 23 (aqueous)	None	April 28, untreated None
			May 3, + NaNO ₃ (0.001N) None
			May 3, + FeCl ₃ (0.00002N) None
	C. May 8 (aqueous)	None	May 14, untreated None
			May 14, + NaNO ₃ (0.001N) None
			May 17, + NaNO ₃ (0.001N) None
			May 19, + NaNO ₃ (0.001N) None
			May 14, + FeCl ₃ (0.00002N) None
			May 17, + FeCl ₃ (0.00002N) None
			May 19, + FeCl ₃ (0.00002N) None

<p>P. caud. 6aII, March 28, 1919</p>	<p>D. May 23 (aqueous)</p>	<p>None</p>	<p>May 26, + NaNO₃ (0.001N)</p>	None
			<p>May 28, + NaNO₃ (0.001N)</p>	None
			<p>May 26, + FeCl₃ (0.00002N)</p>	None
			<p>May 28, + FeCl₃ (0.00002N)</p>	None
	<p>E1. June 6 (aqueous)</p>	<p>None</p>	<p>June 14, + 'tap-water'</p>	None
			<p>June 14, + NaNO₃ (0.001N)</p>	None
			<p>June 14, + FeCl₃ (0.00002N)</p>	None
			<p>June 14, + FeCl₃ (0.00005N)</p>	None
	<p>E2. June 6, 'Na + Ca sol.' (0.01N)¹</p>	<p>None</p>	<p>June 14, + FeCl₃ (0.00003N)</p>	None
			<p>June 14, + 'Na + Ca' (0.01N)</p>	None
			<p>June 14, + 'Na + Ca' (0.02N)</p>	None
			<p>June 18, + 'Na + Ca' (0.01N)</p>	None
<p>P. caud. 17aII + P. aures. 13a, April 5, 1919 (mixed)</p>	<p>A. April 11 (aqueous)</p>	<p>April 12: one pair of caudatum</p>	<p>April 14, untreated</p>	None
			<p>April 25, untreated</p>	None
	<p>B. April 23 (aqueous)</p>	<p>None</p>	<p>May 14, + NaNO₃ (0.001N)</p>	20-30%
			<p>P. caudatum</p>	5%
	<p>C. May 9 (aqueous)</p>	<p>P. aurelia: May 13, casual May 14, casual May 16, 10-20% May 17, 5-10%</p>	<p>P. aurelia</p>	30-50%
			<p>May 14, + FeCl₃ (0.00002N)</p>	Casual
			<p>P. caudatum,</p>	
			<p>P. aurelia</p>	

¹ The 'Na + Ca solution' used in many experiments was composed of 50 parts N sol. of NaNO₃ + 5 parts N sol. of Ca(NO₃)₂ + 2 parts N sol. of NaHCO₃; the dilutions given being expressed in terms of the concentration of the NaNO₃.

TABLE 2

PARAMECIUM STRAIN	DORMANT CULTURE	DATE RENEWED; CULTURE MEDIUM	CONJUGATION IN THE RENEWED CULTURE
<i>P. caudatum</i> , <i>17aIII</i>	March 15, 1919	March 31 (aqueous)	April 10, about 30% April 11, about 20%
<i>P. caudatum</i> , <i>17aIII</i> (<i>P. aurelia</i> , <i>23i</i> added on March 31)	March 15, 1919	March 31 (aqueous)	<i>P. caudatum</i> : April 10, about 20% April 11, about 30% <i>P. aurelia</i> : April 12, one pair
<i>P. caudatum</i> , <i>17aIII</i> (<i>P. aurelia</i> , <i>13a</i> added on March 31)	March 15, 1919	March 31 (aqueous)	<i>P. caudatum</i> : April 10, casual <i>P. aurelia</i> : None

TABLE 3

(Each of the seven cultures of *Paramecium caudatum* listed in the first column was treated as here indicated)

	Dormant since	Conjugation in	Experiments with each race
<i>38b</i>	November 14,	culture <i>2a</i> , <i>44a</i>	as follows:
<i>39a</i>	1919	and <i>57a</i> (p. 351	March 9, + $\text{AlCl}_3(0.00003\text{N})$
<i>44a</i>		of paper)	March 9, + $\text{FeCl}_3(0.00002\text{N})$
<i>57a</i>			March 12, + $\text{AlCl}_3(0.00003\text{N})$
<i>43a</i>			March 12, + $\text{AlCl}_3(0.00009\text{N})$
<i>2a</i>	Renewed March 3,		March 12, + $\text{NaNO}_3(0.001\text{N})$
<i>8a</i>	1920		(Results essentially negative; p. 351 of paper.)

TABLE 4

DORMANT CULTURE	DATE RENEWED; CULTURE MEDIUM	CONJUGATION IN RENEWED CULTURE	SUBCULTURES 24 HOURS AFTER TREATMENT: CONJUGATION
<i>P. aurel.</i> , 13a II, March 28, 1919	A. April 11 (aqueous)	None	April 14, untreated None
	B. April 23 (aqueous)	None	April 25, untreated None
			May 3, + NaNO ₃ (0.001N) 5%
			May 4, untreated Casual
			May 10, untreated None
			May 10, + NaNO ₃ (0.001N) None
			May 10, + FeCl ₃ (0.00002N) None
	C. May 8 (aqueous)	May 13, casual May 14, 3-5% May 16, 10%	May 14, + NaNO ₃ (0.001N) None
	D. May 23 (aqueous)	May 30, casual	May 14, + FeCl ₃ (0.00002N) None
			May 27, + NaNO ₃ (0.001N) Casual
	E1. June 6 (aqueous)	None	May 27, + FeCl ₃ (0.00002N) None
			June 10, untreated None
			June 10, + NaNO ₃ (0.001N) None
			June 10, + FeCl ₃ (0.00001N) None
	E2. June 6, 'Na + Ca sol.' (= NaNO ₃ 0.01N)	June 9, casual	June 10, untreated Casual
			June 10, + 'Na + Ca' (0.01N) Casual
			June 10, 10 cc. culture + 1 cc. FeCl ₃ (0.0001N) 5%
			June 10, 10 cc. culture + 2 cc. FeCl ₃ (0.0001N) 20%

TABLE 5

DORMANT CULTURE	DATE RENEWED; CULTURE MEDIUM	CONJUGATION IN RENEWED CULTURE	SUBCULTURE 24 HOURS AFTER TREATMENT; CONJUGATION
P. aurel., 14aII, April 11, 1919	A. May 13 (aqueous)	None	May 17, + NaNO ₃ (0.001N) May 31, + NaNO ₃ (0.001N) None None
	B. May 26 (aqueous)	None	May 29, + NaNO ₃ (0.001N) May 31, + NaNO ₃ (0.001N) None None None None
	C. June 14, 'Na + Ca sol.' (0.01N)	June 19, casual June 23, casual	May 29, + FeCl ₃ (0.00002N) May 31, + FeCl ₃ (0.00002N) June 19, + 'Na + Ca' (0.01N) June 19, + 'Na + Ca' (0.03N) June 19, 2 cc. culture + 1 cc. FeCl ₃ (0.0002N) Casual Casual
			June 21, + 'Na + Ca' (0.01N) June 21, + 'Na + Ca' (0.03N) 5% Casual
	D1. June 28 (aqueous)	None	June 21, 1 cc. culture + 1 cc. FeCl ₃ (0.0002N) 3% 3%
	D2. June 28, 'Na + Ca sol.' (0.01N)	July 3, 1-3% July 6, casual	July 6, + 'Na + Ca' (0.01N) July 6, + 'Na + Ca' (0.015N) None Casual

TABLE 6

PARAMECIUM STRAIN	DORMANT CULTURE	DATE RENEWED; CULTURE MEDIUM	CONJUGATION IN THE RENEWED CULTURE
<i>P. aurel.</i> , 11 <i>j</i>	July 24, 1919	A. September 19 (aqueous)	None
		B. September 19 NaNO ₃ (0.002N)	September 26, casual
<i>P. aurel.</i> , 11 <i>i</i>	July 24, 1919	A. September 25 (aqueous)	None
		B. September 25, NaNO ₃ (0.001N)	None
		C. September 25, NaNO ₃ (0.002N)	September 30, one pair
		D. September 25, NaNO ₃ (0.003N)	September 30, 3 to 5 pairs

TABLE 7

PARAMECIUM STRAIN	DORMANT CULTURE	DATE RENEWED; CULTURE MEDIUM	CONJUGATION IN THE RENEWED CULTURE
<i>P. aurel.</i> , 1 <i>bI</i> (stock culture)	July 24, 1919	A. September 30 (aqueous)	October 2, 5-10%
		B. September 30, NaNO ₃ (0.0025N)	October 2, casual October 3, 5%
		C. September 30, NaNO ₃ (0.005N)	October 2, casual October 3, 1-3%
		D. September 30, 'Na + Ca' (0.005N)	October 2, casual October 3, casual
<i>P. aurel.</i> , 3 <i>d</i>	July 24, 1919	A. October 4 (aqueous)	None
		B. October 4, NaNO ₃ (0.001N)	October 8, 1-3% October 10, 1-5% October 12, 10-15%
		C. October 4, NaNO ₃ (0.002N)	October 8, 5-10% October 13, 1-3%

TABLE 8

Showing the number of divisions per line for five-day (and ten-day) periods in several lines of *Paramecium aurelia* (11g and 1b) and *P. caudatum* (38a), growing in various culture media (see explanation of tables). The organisms were cultivated on depression slides, renewed daily with fresh culture medium of hay infusion in distilled water, with or without the salts mentioned

PARAMECIUM STRAIN; DURATION OF EXPERIMENT		AQUEOUS MEDIUM						Na + Ca MED. (0.005N)						NaNO ₃ MED. (0.005N)						
		lines						lines						lines						
		1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	
11g	{	1st. 5 days	9	9	9	9	9	13	13	13	13	13								
		2d. 5 days	7	7	8	10	9		9	11	9									
		10 days	16	16	17	19	18		22	24	22									
38a	{	1st. 5 days	6	7				11	10	10	11									
		2d. 5 days	6	4					9	10	10	9								
		10 days	12	11					20	20	20	20								
1b		5 days	4	7	5	7	7	5	10	12	11	11	12	11	11	13	13	10	10	13

Resumen por el autor, John H. Gerould.

Las orugas azules-verdes; el origen y ecología de una mutación en el color de la hemolinfa de *Colias (Eurymus) philodice*.

En Agosto de 1920 aparecieron cuarenta y cuatro orugas azules-verdes de ambos sexos en la progenie de una hembra salvaje blanca de *C. philodice* (bisabuela) cruzada entre sí durante dos generaciones. Las orugas que formaban las generaciones de los abuelos buy padres eran normales, presentando la coloración protectora verde amarillenta. Las mutaciones recesivas azules fueron el resultado de tres cruzamientos de padres verdes heterozigóticos, cuyos descendientes aparecieron en la proporción 3:1. A partir de este momento azul azul produjo la misma clase de descendientes; las formas heterozigóticas verde azul produjeron individuos verdes y azules. Puesto que uno de los progenitores de cada uno de los pares de abuelos era verde heterozigótico, el otro verde homozigótico, es probable que lo mismo sucediese en la pareja salvaje originaria: su cruzamiento entre sí permitió manifestarse al factor latente recesivo. La hemolinfa de la mutación, lo mismo que su piel, es azul-verde. Puesto que el color de la hemolinfa del individuo normal amarillo-verde se debe a un pigmento amarillo (xantofiloide) más un pigmento azul-verde, derivados ambos de la clorofila de la planta que sirvió de alimento, el gene de la mutación (que actúa probablemente desde el núcleo de las células del epitelio intestinal durante la digestión) evidentemente ataca y destruye al primero, dejando al segundo como colorante de la hemolinfa. En la cutícula blanca de la ninfa de color azul-verde, en el ojo de la mariposa y en el huevo recién puesto falta como consecuencia un componente amarillo normal. El color de las alas independiente de la mutación. Los Bracónidos parásitos que emergen de las larvas normales amarillas-verdes tejen capullos de color amarillo vivo; los de las larvas azul-verdes, producen capullos blancos. Los gorriones eliminaron las orugas azules de un cultivo a la intemperie que se dejó descubierto durante doce días, dejando sin molestarlas a la mayor parte de las que presentaban la coloración protectora verde.

BLUE-GREEN CATERPILLARS: THE ORIGIN AND ECOLOGY OF A MUTATION IN HEMOLYMPH COLOR IN COLIAS (EURYMUS) PHILODICE

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ONE TEXT FIGURE AND ONE COLORED PLATE (SIX FIGURES)

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INTRODUCTION

In the course of an investigation into the inheritance of dimorphism (white and yellow female varieties of the butterfly) in *Colias philodice* involving close inbreeding, a remarkable mutation in blood color occurred during the summer of 1920. The mutation is extraordinary in that it affects pigments that are derived without much modification from the chlorophyll of

the food plant and produces a definite change at every stage in the life-history, including the color of the newly laid egg, of the blood and body wall of the caterpillar and pupa, and of the blood and eyes of the adult.

The caterpillar of *C. philodice* feeds upon clover, and is of a yellow-green hue corresponding so closely to that of the plant as to be remarkably inconspicuous. Larvae that are feeding in full view of the observer are almost invisible unless in motion or seen in profile. Although the butterfly is common and I have raised thousands of the caterpillars, I have not found in the open field in the twelve seasons that I have worked upon this species as many, probably, as twelve caterpillars. Imagine, then, my surprise when in August, 1920, caterpillars that were conspicuously blue made their appearance in two of my cultures upon potted clover plants in the greenhouse and my further astonishment when a similar state of affairs was soon afterwards discovered in an outdoor culture upon a series of clover plants covered with cages.

Self-colored blue, or more accurately blue-green, caterpillars are almost unknown in Lepidoptera. Only two species among diurnal butterflies have as yet come to my notice, both European pierids, *Anthocharis cardamines* and *Pieris ergane*. None of the species closely related to either, so far as I know, are blue-green, so that it is possible that each has experienced in its ancestral lineage a mutation like the one here described for *Colias philodice*, also a pierid. Toyama ('12) mentions the occurrence of blue silkworms, "a Mendelian characteristic and recessive to the normal-skinned characteristic," due, as he states, to the absence of pigment in the hypodermis.

The color sensation that the mutant caterpillars give one against the food plant as a background is like that produced by blueberries on a bush, and it was only by comparing them side by side with blueberries (*Vaccinium*) that I first convinced myself that they are slightly more green than blue. 'Light porcelain green' of Ridgeway's ('12) Color Standards (pl. 33, *B-G*), classed as 'blue-green,' corresponds closely to the ground color of the full-grown caterpillar.

The evidence regarding the origin of this recessive mutation points to the conclusion that it can be traced back to an individual heterozygous for it, and that it was brought to light by two successive acts of inbreeding, of which the first was a homozygous dominant \times heterozygous dominant, and concealed the mutation. By good fortune, however, three out of a total of six matings of the next generation (bred for an entirely different purpose, for blue caterpillars were then unknown) turned out to be combinations of heterozygote \times heterozygote and revealed the blue-green recessives.

It is a remarkable fact that the mutation affects all the four stages in the life-history. The egg cytoplasm at the time of laying is pure alabaster-white instead of cream-white; the larvae are blue-green, the blue color persisting, though less strikingly, through the pupal stage; the pupal cuticula is white instead of pale brownish-yellow, and the eye color of the adult is bluish 'mineral green' rather than the more strongly yellow 'apple green' of the normal eye. This is the first case, so far as I know, in which eye color in insects is known to be intimately connected with blood color, and in this case probably directly determined by the physicochemical nature of the hemolymph.

That the blood-color of the pupa determines the color of its cast cuticula, turning it white, is corroborated by the fact that the silk spun by certain parasitic Hymenoptera (Braconid larvae) emerging from a blue-green caterpillar is white, instead of golden-yellow, the normal cocoon-color of the same species of parasite feeding upon normal yellow-green caterpillars.

This mutation strengthens the evidence that green caterpillar color is not unmodified dissolved chlorophyl, but that the latter in being absorbed through the wall of the intestine into the hemolymph undergoes certain not very profound changes, as it combines with the proteids suspended in the plasma. In the present case the yellow component of chlorophyl, xanthophyl, is evidently broken down or decolorized by a recessive gene, or hereditary chromosomal enzyme, acting locally from the nuclei of the cells of the intestinal epithelium upon the digested leaf-green during its absorption into the hemolymph. The recessive

gene probably destroys xanthophyl, but leaves intact a pigment derived from the blue-green component of chlorophyl proper, called chlorophyl *a*. The nature of the physicochemical change involved, however, will be the subject of further investigation.

All the recent investigations into the chemistry of green pigments in the hemolymph of plant-eating caterpillars that have come to the writer's attention (e.g., Dubois, '09; Steche, '12; Geyer, '13, and Przibram, '13) support the conclusion of Poulton ('85, '93) that these green and yellow pigments are absorbed into the blood without undergoing fundamental changes. The different pigments, however, xanthophyl and chlorophyllin (chlorophyls *a* and *b*) are not absorbed in equal degree in the two sexes, according to Steche ('12) and Geyer ('13) for, while the blood of the female receives both chlorophyllin and xanthophyl in proportions similar to those that occur in the normal leaf and is consequently in certain species greenish, that of the male of the same species contains only modified xanthophyl, or else none of these pigments whatever, and is accordingly yellowish or colorless. Thus, presumably, in the male the cells of the walls of the intestine produce a metabolic reaction upon these pigments, destroying chlorophyllin and in some cases xanthophyl also, while the corresponding cells in the female have no such effect. That this breaking up of the green pigment in the male is not due to an enzyme in the hemolymph itself, and accordingly should be ascribed to the action of the intestinal epithelium, is shown by the fact that mixing the blood of the male with that of the female does not decolorize the latter.

The blue-green mutant caterpillars of *C. philodice*, however, are equally distributed between the two sexes, and the same is true of grass-green larvae heterozygous for blue-green. In this case xanthophyl is destroyed, not chlorophyllin, and the decolorizing gene is not sex-limited. Whether a sex-difference exists between the blood pigment of the male and female in *C. philodice* is still unknown to me. In the common cabbage butterfly,¹

¹ A series of observations on *P. rapae* that I have recently made show well-marked individual variations such as Guyer has described, but those that I observed were not sex-limited.

Pieris rapae, however, a somewhat closely allied form, Geyer found the hemolymph of the male caterpillar to be light yellow, that of the female, bright green.

The blue-green caterpillars had been under observation only about three months when this paper was written, but the facts regarding the origin of the mutation, the ecological data already obtained, the many-sided relations of the blood pigments and the action upon them of the mutant gene, are so striking and interesting that it seems best to put the case on record as it stood at the close of the first season. I hope later to present a more complete account of the matter, following out some of the many lines of research that are hardly more than pointed out in the present paper.

I wish to acknowledge my deep indebtedness to Prof. A. Ames, to whose skill the exceedingly accurate record of colors upon the plate is in large measure due; to Prof. Leland Griggs and Mr. Kenneth Robes for the photographs themselves; to Mr. A. B. Gahan, of the National Museum, who identified the hymenopterous parasites that attacked the blue caterpillars; to Mr. Rolf C. Syvertsen, who assisted me in rearing the caterpillars and protecting them against disease, and to Mr. C. J. Lyon for assisting me in acquainting myself somewhat with the chemistry of chlorophyl.

I extend my most appreciative thanks to the authorities of The Wistar Institute for providing a plate in colors. Funds necessary for the prosecution of this research were generously furnished first by the President and Treasurer of Dartmouth College, and later by the Carnegie Institution of Washington through the kindness of Dr. A. G. Mayor, to all of whom I make grateful acknowledgment.

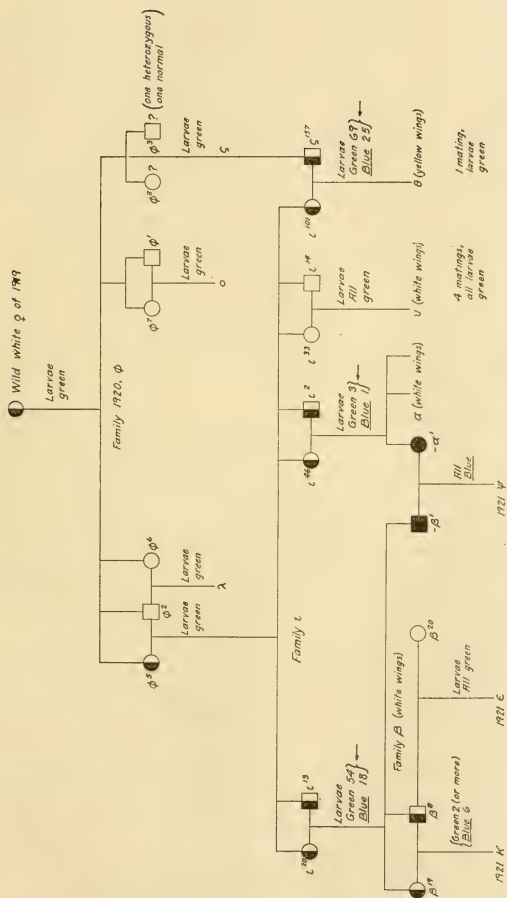
ORIGIN OF THE MUTATION

The fact that the blue-green caterpillars first appeared in rather large numbers (25 in one family, θ , 18 in another, β , text fig. 1) in the same generation of inbred stock, and comprised in each case approximately 25 per cent of their respective families or

broods, shows at once that their parents all were heterozygous for the new character. Moreover, since these heterozygous grass-green-blooded parents were brothers and sisters (family 1920, ι) with the exception of one male (s^{157}) which was a cousin, we must look to the previous generation of adult butterflies (1920, ϕ) for the individuals in whose germ cells the mutation may have occurred.

The grass-green caterpillars that became this previous generation were the hibernating offspring of a white-winged female of the preceding autumn. Of the seven individuals of this family that were used in breeding, at least two (ϕ^5 ♀, white-winged, and either ϕ^8 ♀, yellow-winged, or her mate ϕ^3 ♂) were heterozygotes for blood color, but, mating in each case with normal homozygous dominants for blood color, no recessives appeared in their immediate offspring (1920, ι , s). It seems probable, furthermore, that other heterozygotes for the blue mutation may have existed in this brood, ϕ , making up probably 50 per cent, but the offspring of only one pair (ϕ^5 ♀ \times ϕ^2 = ι) were thoroughly tested (giving broods β , α , θ , ν). Two other matings (ϕ^6 ♀ \times ϕ^2 ♂ = λ , and ϕ^7 ♀ \times ϕ^1 ♂ = o) were made, one of which, λ , was carried no further, while two couples from the other produced only grass-green caterpillars.

Since more than one and possibly 50 per cent of brood ϕ were heterozygous for blue, it is probable that the mutation should be traced back to the wild female of the autumn of 1919 or to her mate, as the original heterozygous mutant, else we must suppose that the similar mutation occurring by hypothesis in two of her offspring originated quite independently of each other and of the parental genotype, which would be quite contrary to general principles in heredity. The case illustrates well how a recessive mutation may be transmitted by heterozygotes, outcrossing with normal dominants for many generations, without coming to light. There is reason to suppose that this is often the case under natural conditions and that close inbreeding of the wild stock for a few generations is all that would be necessary to demonstrate the existence of these latent recessive factors.



Text-fig. 1 Origin of the blue mutation. Circles represent females, squares males. Half-shaded symbols are individuals from normal grass-green caterpillars that are heterozygous for the recessive blue-green. Fully shaded symbols = homozygous recessive individuals, 'blue', i.e., blue-green, as caterpillars. Unshaded symbols = individuals from normal caterpillars, either homozygous grass-green dominants or grass-green heterozygotes of unknown genetic constitution.

EFFECT OF THE MUTATION ON THE EGG

The first sign of the mutation in the life-cycle appears in the cytoplasm of the newly laid egg of a female homozygous for blue-green, which is pure white, like alabaster, rather than the normal cream-white. Thus from the start the protoplasm of the embryo lacks a yellow element. This abnormality in egg color was not observed in the heterozygous stock, but it was found to be true of all the eggs laid by a female that had been a blue caterpillar and on eclosion had been mated to a male that also had been a blue-green larva; that is, these were the fertilized eggs from a pair of recessives with blue-green hemolymph.

The eggs laid by a female heterozygous for blue-green are cream-white, the color of the newly laid egg being determined directly by the blood color of the mother, which appears to be the general rule in Lepidoptera.²

As the reader will presently be shown, and at this point must bear in mind, the basis of normal yellow-green blood color is obtained directly from the food. The inherited differential factor in the mutant is a catalyst that destroys xanthophyl rendering the blood blue-green instead of yellow-green and thus *indirectly* changing the eggs from cream-white to pure white.

During the first twenty-four hours after being laid, the egg, now pale (cream-white or pure white), is going through its early cleavage stages. Presently (probably upon the formation of the blastoderm) it turns red, whereupon, so far as my observations extend, a sharp distinction between the egg of grass-green and blue-green embryo caterpillars is not visible. If then, one of the two maturation divisions is differential, any egg of a female heterozygous for blue-green has an even (50:50) chance of being, at the moment of fertilization and laying, a potential blue-green caterpillar, its nuclei having received the recessive gene that inhibits the further development of yellow. It seems improbable that in the brief period following egg-laying before the now fertilized egg turns red (cleavage stages) the relatively few nuclei of a homozygous embryo from a heterozygous mother

²Repeated observations made during the following season (1921) confirm these statements.

could decolorize the relatively large mass of yolk-filled protoplasm presumably at the time cream-colored, and make it appear like the egg of a homozygous female pure white.

Moreover, Toyama ('13) has shown that in the silkworm not only the color of the newly laid egg, but even the color of the serosa (containing nuclei that combine both paternal and maternal genes) show only maternal inheritance. The action of the paternal gene is entirely neutralized or masked in reciprocal crosses by some maternal effect, which, in the opinion of the present writer, must lie in the composition and action of the maternal blood.

BLOOD AND BODY COLOR OF THE LARVA

The mutation is not evident during the first stage of larval development, when the skin is relatively opaque, nor until the end of the second. At that time, just before the second molt when the skin is tightly stretched, the young larva becomes distinctly bluish, and after the second molt conspicuously and even brilliantly blue-green.

Full-grown caterpillars (pl. 1, fig. 1) correspond in color to 'light porcelain green' (Ridgeway, '12, pl. 33, *B-G*) above and 'Niagara green' below, running into 'Montpelier green' on the head, which is slightly darker. I am indebted to Prof. A. Ames, an artist and authority in optics, for assistance in this determination. Another mature caterpillar was a shade darker, 'Montpelier green.'

A distinctive feature of the blue-green caterpillar is the entire absence of the pink line that in a normal larva is traced more or less plainly along the middle of the lateral white band running through the stigmata. The pink lateral line of the normal larva is, therefore, a dominant character completely correlated with grass-green hemolymph.

COLOR OF THE PUPA AND OF ITS CUTICULA

The pupa (fig. 2) is somewhat less blue than the larva, but its hypodermal color is a well-marked discontinuous variation from the normal grass-green hue (fig. 2a). Its ground color corresponds to a blue-green called 'Rejane green' by Ridgeway ('12, pl. 33, *GB-G*.)

The cuticula of the pupa, shed by the eclosion of the adult (fig. 3, cf. fig. 3a) is white instead of yellow. The blue-green hypodermis of the pupa, deprived of the normal yellow pigment, secretes a white rather than a yellow cuticula.

Observations made in 1921 confirm this, though the posterior abdominal segments of the cast cuticula in some individuals is tinged with brown, probably from excrementous pigments voided by the butterfly before eclosion, and the anterior half of the pupal shell including the wing cases is regularly *faintly* yellowish and by transmitted light slightly greenish, as shown by figure 3.

IMAGO AND ITS EYE COLOR

Color changes in the wings of a butterfly from such striking mutants were to be expected, for it is well known from the work of Mayer ('97) and others that the blood that flows into the hollow scales shortly before eclosion provides the pigments of the wing colors, yet no noticeable change in the hue of the scales was produced by the mutation. Thus the two broods containing 25 per cent of blue-green caterpillars, viz., 1920, α and β (text fig. 1), were homozygous for white wing colors, while a third, θ , was homozygous for yellow. α and β came from white mothers and brother-sister matings within the large family ι [that included 95 white, 28 yellow females, or 3:1]. The relation between caterpillars and adults within family β was as follows: the 29 grass-green caterpillars that reached maturity became ' β ' adults = 18 white ♀♀, 11 ♂♂; the 12 surviving blue-green caterpillars became ' $-\beta$ ' adults = 6 white ♀♀, 6 ♂♂. θ , on the other hand, came from a yellow mother of this same family, ι^{101} ♀, mated with a male of pure yellow stock, s^{157} ♂, and the 33 mature offspring were all yellow (10 ♀♀, 17 ♂♂ from grass-green larvae, viz., θ , and 3 ♀♀, 3 ♂♂ from blue-green larvae, viz., θ).

The blood of the imago, as seen by bleeding an emerging butterfly, continues to be blue-green. It still lacks the yellow element that would have been introduced into it from the food had it not been, in all probability, neutralized or decolorized by an inhibitor. It is evident that this inhibitor of yellow

pigment has no effect whatever upon the yellow or the white wing pigments that appear in the imago. The latter, while just as literally in the blood as are the former, are chemically quite unlike them. Xanthophyl, from which the yellow element in the normal caterpillar's blood is derived, is said to have the formula $C_{40}H_{56}O_2$, while the yellow pigment of *Colias*, as Hopkins ('94) has shown, is allied to uric acid, $C_5H_4N_4O_3$.

The eye color of an adult from a blue-green caterpillar (fig. 4, cf. fig. 4a) is strikingly affected by the mutation. In every case there is a noticeable lack of yellow. The eyes of the mutant are 'mineral green' (Ridgeway, '12, pl. 18, 31' *Y-G*), rather than 'apple green' (pl. 17, 29' *GG-Y*). In some mutant individuals this color difference is very marked, while in others it can be detected only by comparing them closely with an individual from a grass-green caterpillar. This change in eye color is probably an effect produced directly by the altered blood deprived of xanthophyl.

This case supplements the principle brought out by Morgan et al. ('15, p. 32) that a single mutation may affect many parts of the adult body, inasmuch as this mutation affects every stage in the life-history. It is, among other attributes, a recessive mutation in eye color comparable to those in *Drosophila*. The same yellow element is lacking in the hemolymph and compound eye that is also missing in the egg, in the blood and body color of the caterpillar, and in the cuticula of the pupa.

DISCUSSION

Since the blood, or hemolymph, permeates all the tissues and is absorbed into the hypodermal cells, one is tempted to believe that we have here a mutation that manifests itself primarily in the blood, and that the hypodermal cells are blue-green and secrete white rather than yellow cuticula because permeated with the non-yellow hemolymph. If this conclusion is justified, the mutations in eye color in *Drosophila* also may be due to changes in the blood plasma, and when the nature of these physicochemical changes is ascertained we shall be a step nearer a knowledge of genes themselves.

But in this view we must not lose sight of the chromosomes which the behavior of the spermatozoon in fertilization shows to be practically the exclusive vehicle of paternal factors and should carry in the egg the corresponding genes of the maternal parent. It is, therefore, to the chromosomes that we must look for the determiners of blue-green color. Logically, following the chromosome hypothesis, one must then suppose that the nuclei of hemopoietic cells or more probably those of the intestinal epithelium concerned with the digestion of chlorophyll carry the gene that determines the color of the blue-green blood.

No one would claim, however, that the physical basis of normal blood color in a grass-green caterpillar is inherited. As Poulton ('85) has shown, it is a substance derived from digested chlorophyll absorbed from the intestine, and is, both physically and chemically, akin to chlorophyll. The recessive mendelian factor involved is probably an inhibiting enzyme capable of neutralizing a yellow element of this plant-derived pigment diffused in the hemolymph, leaving it blue-green.

EFFECT OF BLUE-GREEN BLOOD ON THE COCOON-COLOR OF A PARASITE

That the blood of the pupa permeates the tissues sufficiently to act directly upon the hypodermis and cuticular secretion, and to determine their color independently of any possible local action of hereditary enzymes from the hypodermal nuclei, was shown conclusively by the fact that the silk spun by braconid parasites living in blue-green caterpillars was white (fig. 5), while that produced by others living in grass-green caterpillars was bright-yellow (fig. 5a).

This was demonstrated by an experiment that performed itself in an interesting outdoor culture, 1920 θ . The caterpillars of this brood were either grass-green or blue-green (in the 3:1 ratio). Several caterpillars of each color-form were attacked by a braconid (fig. 6), *Apanteles flaviconchae* Riley³. The

³ Kindly identified for me by Mr. A. P. Gahan, of the National Museum, who "finds no difference between the specimens which spin the bright yellow cocoons and emerge from the 'green' larvae and those which spin the white cocoons and emerge from 'blue' larvae."

larvae of this parasite rapidly develop within the tissues of the caterpillar and come to the surface of the skin, where each immediately spins a silken cocoon, normally of golden yellow. To my great surprise the braconid cocoons upon the surface of the parasitized blue-green caterpillars were not bright yellow, but pure white. After feeding upon the blue-green, yellow-free blood of the host, the secretion of their silk glands had become pure white.

Unlike white cocoon color in *Plusia moneta* (Bird, '03; Hawkes, '16) and *Clisiocampa neustria* (Hawkes, '16) that turns yellow in a moist atmosphere, white in the cocoons of *Apanteles* emerging from a blue caterpillar remains unchanged in the presence of moisture.

CONCLUSION

It is exceedingly probable that normal yellow silk of *Apanteles flaviconchae*, parasitic upon normal yellow-green caterpillars, gets its color from a xanthophylloid element in the h molymp, derived from the food plant. This the blood of the blue-green caterpillar lacks. Consequently, the parasite of the blue-green caterpillar spins white, instead of yellow, silk.

DISCUSSION

There is in this case no opportunity to appeal to chromosomes of the spinning glands of the braconid larvae for an explanation of the yellowness of their product. The yellowness is due directly and absolutely, so far as my observations go, to the blood of the host and not to hereditary enzymes in the chromosome of the spinning glands.

That being so, we must infer that a similar explanation holds for the grass-green of the hypodermis, yellow color of the pupal cuticula, and for adult eye color, of the host, *Colias philodice*, for they show a similar relation to blood pigments in that if yellow is suppressed in the blood it will be lacking in the very permeable hypodermal cells and their secretions; that is, in the cuticula of the pupa and in the compound eye. Here, too, we may disregard the possible local action of the chromosomes

for we have a more plausible explanation in the direct action of the blood.

If it can thus be shown that hereditary characters are directly determined by the physicochemical nature of the blood, and only indirectly and *ab initio* from the physicochemical action of chromosomes, we have taken a step toward the solution of the problem of the nature of mendelian factors, for the hemolymph of a caterpillar, like the blood of the higher animals, is subject to chemical and physical methods of analysis, and we may be able ultimately to understand and control the development of mendelizing and other characters that become in a new sense 'in the blood.'

HOW LAMARCKIAN FACTORS MAY REACH THE GENES

Nor should we neglect the study of the blood in its determinative effects upon development in investigating the possibility of permanent lamareckian adaptive changes. Is the blood susceptible to physicochemical changes due to the environment that may, on the one hand, determine the course of development and, on the other, produce a corresponding change in the chromosomal enzymes, or genes, in the germ cells? If that is possible, then the blood of the offspring would react like that of the parent in the determination of development and a lamareckian mutation would be inherited.

It is beyond the scope of this paper to gather up and consider evidence for and against this view, but the reader is asked to take with some reserve Weismann's idea which since 1890 has become a biological dogma that the germ cells are so isolated from the soma and the world that their chromatin cannot be specifically changed by environmental influences, that no mechanism exists for the transfer to the germ cells of specific environmental effects that are observable in the soma. The blood, or hemolymph, in the writer's opinion, is such a mechanism. It is susceptible, especially in caterpillars, to chemical changes produced by the food. Are the chromosomes of the germ cells immune to all such changes?

That they are not is urged by Harrison ('20), who ascribes the increasing prevalence of melanism of moths near English industrial centers to "changed metabolism favoring resistance to, or actually induced by, the use of food contaminated with metallic salts and other compounds." Melanism in moths of the genus *Oporabia*, however, upon which his paper treating of the possible inheritance of acquired melanism is based, unfortunately, is non-mendelian in its inheritance. A persistent blend occurs. In other words, melanism has not affected the chromosomes, though it apparently has affected the hemolymph. The next step would be to show that the hemolymph may in turn induce mutation in the chromosomes.

We may well be skeptical of 'proofs' of the inheritance of acquired mutations that are not checked up by control experiments running through more than one generation, for the origin of the blue caterpillars shows that to reveal the nature of the genes and bring out latent recessive mutations that might be ascribed to environmental factors, two or more generations of inbreeding may be necessary.

Suppose, for example, that a culture under the stress of environmental conditions should prove to contain a new recessive mutant and that a control culture of the same stock bred under normal conditions apparently does not. The usual presumption has been that the new variation has been produced by the environmental factors. The presumption, on the contrary, should be that the experimenter is working with heterozygous material, that a pair of heterozygotes have in fact produced his mutants, and that his control culture, being made up of heterozygotes mated with homozygous dominants and apparently composed exclusively of normal individuals, should be further inbred to show its real genetic constitution. No such carefully controlled experiments giving positive reliable results have yet come to the writer's attention.⁴

⁴ At the time these words were written Dr. M. F. Guyer's and E. A. Smith's very important paper on the inheritance of an acquired eye defect in rabbits had not been read (vol. 31, no. 2 of this Journal). It should have been included in this discussion.

On the other hand, Federley ('20) has recently analyzed the case of supposed mutative effects of cold in Fischer's ('01) experiments with *Arctia caja*, and shown that the supposed positive results that have often been quoted as proof of the inheritance of acquired characters are in all probability due to the heterozygous nature of the stock.

I concur entirely with his conclusion that wild species frequently are only apparently constant, but really heterozygous for a considerable number of genes, and have emphasized this in an essay (Gerould, '14) calling the attention of systematists to the importance of testing species whenever possible by inbreeding and cross-breeding them. Federley, furthermore, makes the interesting suggestion that 'varieties' are groups of individuals showing rare combinations of genes (extracted recessives from the heterozygous type), while 'aberrations' are still rarer combinations from complexes of polymeric factors.

ACTION OF ENVIRONMENTAL FACTORS AND GENES UPON CERTAIN BLOOD PIGMENTS

There can be little doubt that the blood plasma plays an important part in the ontogenetic development of seasonal variations, which in some cases are as strikingly abrupt as mutations. An interesting example of this class of variations has recently come to the attention of the writer. Two forms of *Leptalis spio*, a pierid butterfly, occur in Porto Rico, specimens of the generation emerging in May being marked with orange, those of the July brood with yellow,⁵ the latter but not the former being an excellent 'mimic' of *Heliconius charitonius*. It would be interesting to discover the relation of pigments in the hemolymph to this striking change. Climatic changes in radiant energy and moisture produce periodically and rhythmically in cases like this a reversible chemical reaction in blood pigments involving a visibly abrupt variation. The seasonal difference may be said to be 'qualitative,' though it is brought about by a quantitative difference in temperature or moisture.

⁵ To Mr. F. E. Watson, of the American Museum of Natural History, I am indebted for the idea and the evidence that this is a case of seasonal dimorphism.

The degree of intensity or, in musical terms, 'pitch' of the seasonal variations of *Colias eurytheme* that I have studied and shall describe in another paper is to be explained by the direct action of environmental factors upon the blood, restraining or forcing on the chemical elaboration of orange pigments that the hemolymph lays down in the wing scales. At the same time these butterflies are subject to much individual variation due to the action of genes.

In this case, as in the subject of the present paper, the substrate upon which the chromosomal enzymes act to produce individual variations is not inherited, nor does it come from the food. It is derived from the uric acid that has accumulated during the pupal stage, which, according to Hopkins ('96) furnishes the yellow and orange pigments of *Colias*.

While these pigments, or this pigment, are, strictly speaking, not inherited, they are under the direct control of genes, for I have found that orange and yellow in the species-cross *C. eurytheme* \times *philodice* segregate through the action of two pairs of homomeric multiple factors in true mendelian fashion, enormously increasing the range of variation in F^2 over that in F^1 , but without abrupt discontinuity. Simultaneously the elaboration of the same orange pigment is in high degree under environmental control, especially that of temperature. What, then, is the rôle of the mendelian factors involved in orange vs. yellow wing color? They are presumably enzymes that produce a reversible chemical reaction in this particular blood pigment, as a dominant oxidase vs. its absence or a recessive reductase. The intensity of this chemical reaction (oxidation?) taking place in every individual of a given seasonal generation is practically uniformly controlled by the climatic conditions of that season, toning up or shading down the orange hue of every individual, but not determining individual variations. The evidence supporting these conclusions must be reserved for detailed treatment in a future paper.

To summarize: in this cooperative action of climate and genes upon the oxidation of these urates in the blood, climate regulates the intensity of the reaction nearly uniformly in every individual

of a given generation (cf. the 'pitch'); genes act differentially upon individuals, determining the particular intensity exhibited by each as compared with its brothers and sisters.

INADEQUACY OF THE PRESENCE-ABSENCE HYPOTHESIS TO EXPLAIN GRASS-GREEN VS. BLUE-GREEN BLOOD COLOR

The reader is now asked to return from this digression to the main subject of this paper, pigments derived from chlorophyll, which have little in common with the yellow-orange urates just discussed except that both are dissolved in hemolymph.

A xanthophylloid yellow element is present in the blood of the dominant grass-green (body and blood color) caterpillar and absent from the recessive blue-green, but it is clearly evident that the dominant character grass-green is produced not by the dominant gene, as the presence-absence theory would imply, but is supplied by the food. We are safe, I believe, in accepting the conclusion of Poulton, Mayer, and later observers that the yellow component of green blood pigment in plant-eating caterpillars is modified xanthophyl that has left the plastids of the food plant and combined with proteids in the blood plasm. The active factor in this pair of allelomorphs is the recessive, an inhibitor or decolorizer that attacks and destroys xanthophyl on its way into the hemolymph, or otherwise suppresses the xanthophylloid pigment of normal grass-green blood. The dominant hereditary factor representing the normal grass-green is probably in this case a blank, an absence of the recessive gene, for the physical basis of the dominant yellow-green is furnished by the food. Thus, to fit this case, the presence-absence hypothesis must be inverted.

ELIMINATION OF THE BLUE-GREEN MUTANTS BY NATURAL SELECTION

English sparrows in the summer of 1920 were a serious hindrance in my breeding experiments, boldly entering the greenhouse through the open sash and feeding on caterpillars or pupae that could be reached by pecking through the screening of the cages. Outdoor cultures suffered still worse from the ravages of the birds.

Brood θ , which consisted altogether when brought into the laboratory of sixty-nine grass-green and twenty-five blue-green caterpillars, came from three successive layings outdoors, of which nos. 1 and 3 were continually covered with cages, while no. 2 on a spot close to the others was left uncovered from August 4th to 16th, owing to a lack of cages and because when setting the culture I had no great interest in it (the brood being expected to be homozygous for the common yellow-wing form). On August 16th blue-green caterpillars were discovered greatly to my surprise in cages nos. 1 and 3. Between a third and a quarter of the caterpillars in these cages were blue-green. On the spot where cage no. 2 had stood during the laying of the second lot of eggs, and which for twelve days had been uncovered and open to the attacks of birds, almost exclusively grass-green caterpillars in various stages of development were found. By careful searching I succeeded in finding two very small and not yet conspicuous blue-green larvae that had escaped the eyes of the sparrows. It was perfectly evident that the birds had found the blue-green mutants an easy mark and that, while leaving plenty of grass-green caterpillars untouched, they had eliminated from that part of the culture nearly every blue-green individual.

INHERITANCE OF THE MUTATION

The evidence that the blue-green mutation is inherited as a mendelian recessive is not extensive, yet definite and satisfactory as far as it goes.

As explained above in the section describing the origin of the mutation, the first three broods in which it appeared were from parents all of which were grass-green heterozygous dominants. These broods are shown in detail in the following table.

Inspection of table 1 shows that in the first generation the 3:1 ratio between grass-green and blue-green larvae was closely approached in the total number of individuals that came through to the imago, and that, in the census of all the larvae, both living and dead by disease, in each of the three families, the same proportion is evident. It will also be seen that the numbers

TABLE 1
First generation showing the mutation

FAMILY	FEMALE PARENT	MALE PARENT	GRASS-GREEN LARVAE		BLUE-GREEN LARVAE		NOTES
			♀ ♀	♂ ♂	♀ ♀	♂ ♂	
1st							
α	♂ ^{46*}	♂ ²	1*	2	1*	0	Mated July 21. Eggs mostly sterile. First laying July 24, hatching July 30, pupation August 20, eclosion September 2.
β	♂ ^{20*}	♂ ¹³	18*	11	6*	6	Mated July 21. First blue-green larvae observed August 9 in 2nd-3rd stage. Pupation began August 15, Eclosion began August 23, Census of larvae on August 22:
							<div>Living Dead Actual Expected</div> <div>Grass-green.....50 9 59 [57½]</div> <div>Blue-green.....17 1 18 [19½]</div> <div>Total.....77</div>
θ	♂ ^{101†}	♂ ¹⁵⁷	10†	17	3†	4	Mated July 26. Set July 28. Eggs July 29. First grass-green pupa August 21, blue-green August 22. Eclosed August 30, September 2. Census of larvae on August 22:
							<div>Actual Expected (3:1)</div> <div>Grass-green.....69 [55½]</div> <div>Blue-green.....25 [18½]</div> <div>Total.....74</div>
Totals.....			29	30	10	10	
Grand total.....			59 grass-green + 20 blue-green				
Expectation.....			59½ grass-green + 19½ blue-green				

* White wings.

† Yellow wings. The male always has yellow wings.

of blue-green individuals are equally distributed between the sexes.

In the second generation two 'blue-green' parents gave only blue-green offspring (family ψ) and the two heterozygous 'grass-green' gave both dominant and recessive varieties, though in numbers too small to be of significance.

TABLE 1—Continued
Matings giving a second generation

FAMILY	FEMALE PARENT	MALE PARENT	LARVAE	NOTES
1920-21				
ψ	—α ¹	—β ¹	All blue-green	Both parents were blue-green as larvae. Mated September 2. Few eggs laid on September 9, pure white, not cream white. The few laid were mostly fertile. Female inactive and weather conditions unfavorable to incite egg-laying. She died September 27. 5 larvae, <i>all blue-green</i> , in hibernation.
κ	β ¹⁹	β ⁸	Grass-green and blue-green	Both parents grass-green heterozygotes, but the small brood of caterpillars contained an excess of blue-green, viz., 6 blue-green and 2 grass-green (probably not all the grass-green larvae were seen, though they were much in excess of 25 per cent blue-green). Caterpillars in hibernation. β ⁸ ♂ was again mated with another sister, β ²⁰ ♀ giving a large brood, ε, of grass-green larvae. ¹ Since this is a DR × DD combination, inbreeding next summer is expected to bring out plenty of blue-green larvae.

¹ (Postscript, September, 1921.) From this brood, ε, during the summer of 1921 blue-green caterpillars were recovered, but only by mating females of ε to wild males and then inbreeding, as will be described in a later paper.

[Note added September, 1921: These facts regarding the inheritance of blue-green have been amply supported by the experiments of the following season (1921), when a new recessive mutant caterpillar, olive in color, producing a butterfly with olive-colored eyes, appeared in recovering the blue-green strain from grass-green after back-crossing with wild stock.]

VIGOR OF THE MUTANT CATERPILLARS

The census of brood β as caterpillars, viz., 59 grass-green: 18 blue-green, was also perfectly definite and tends to show that the blue-green larvae are stronger and more resistant to disease

than the normal yellow-green. Possibly this will explain the excess of blue-green individuals in brood θ , notwithstanding the inroads upon one part of it by English sparrows, as described in the previous section. A similar excess of blue-green caterpillars was noted in the small family of the second generation, κ , as shown in table 1. The small size of the brood is due to infertility that accompanied inbreeding, rather than to disease. If the blue-green caterpillars should prove to be stronger and more resistant to disease (especially the virulent polyhedral disease with which the breeder of lepidoptera constantly contends), the conspicuousness of its color is a handicap that would never allow it to succeed in the struggle for existence, unless it should find and adapt itself to a bluish-green food plant of its favorite botanic family, the Leguminosae.

SIMILAR PIGMENTS

The pronounced blue-green hue of the caterpillar, however, is not exactly matched by any pure leaf color that I know. It is physically comparable in the vegetable kingdom to the tint of the blue-green algae, which is due to a special blue pigment (phycocyanin) in addition to chlorophyl. Phycocyanin, according to West ('04), is a "reserve albuminous substance containing both nitrogen and phosphorus, and it occurs in small granules."

The source of the blue-green color of the mutant caterpillar is the blue-green component of chlorophyl from the food plant, if, as I assume, the yellow component xanthophyl, or its deoxidized derivative carotin, is destroyed, or at least changed and decolorized, during digestion, leaving only the blue-green component. The latter, formerly called chlorophyllin, is now known through the researches of Willstätter ('13) and others to be a mixture of two pigments: one of which, chlorophyl *a*, $C_{55}H_{72}O_5N_4Mg$, constituting about 72 per cent of the mixture (with a variation of not over 10 per cent) is blue-green in alcoholic solution as seen by transmitted light, blood-red by reflected light; the remaining 26 per cent is called chlorophyl *b*, yellow-green by transmitted light, differing from its associate in the lack of two atoms of hydrogen and the addition of one of oxygen.

It is of course not possible to say at present that the blue-green pigment of the blood and integument of the mutant is identical with chlorophyll *a*, for certain changes may have occurred in digestion of the chloroplastids and recombination of the pigments with proteids dissolved in the hemolymph, but Poulton's ('85) studies show that spectroscopically the pigments of normal green caterpillars appear to have suffered no fundamental change from the mixture of chlorophyll and xanthophyll that make up ordinary leaf-green.

Dr. William Patten, on looking at the mutant caterpillars, was immediately impressed with the similarity between their color and that of well-oxygenated hemocyanin as seen in the blood of *Limulus*. A close comparison between the two was accordingly made. It was found that the greenish hue that occurs in the mutant caterpillar's blood is lacking in hemocyanin though both, until brought together for comparison, look much alike.

The resemblance is interesting, in view of the well-known chemical similarity of the animal and plant pigments concerned with respiration and photosynthesis. Hemocyanin, hemoglobin, and chlorophyll are chemically closely related. They have definite chemical interactions with CO_2 and O, and their decomposition products, hematoporphyrin from hemoglobin and phylloporphyrin from chlorophyll, are almost identical. The possibility is at least worth investigating whether the chlorophylloid pigments of the blood of caterpillars, though not respiratory in the same sense as hemoglobin and hemocyanin, may not perform some function in connection with the elimination of CO_2 . The caterpillar's hemolymph is acid, as Poulton ('85) and Mayer ('97) have shown, which may be due to the presence of CO_2 . Moreover, Mayer pointed out that in an atmosphere of CO_2 the hemolymph does not coagulate. There is no direct positive evidence, however, known to the present writer that this green blood pigment is a reducer of CO_2 or performs such a supplementary respiratory function as that just suggested. If such a reaction occurs, it cannot depend, as in photosynthesis,

upon the presence of light, for many green caterpillars feed and thrive well though enclosed in a box or jar from which light is excluded. This species, however, feeds only by daylight.

FACTORS AFFECTING THE COLOR OF BLOOD AND COCOON IN THE SILKWORM

Yellow blood in silkworms is closely correlated with the spinning of yellow silk, white blood with white silk. Ude ('19), however, has discovered a strain of yellow-blood stock that spins white silk, though their silk glands are yellow.

A homozygous mutant, i. e., yellow blood, white silk \times white blood, white silk, if the latter is a genuine double recessive, gives only the double dominant yellow blood, yellow silk in F_1 , which, inbred, produced 9 yellow blood, yellow silk, 3 yellow blood, white silk, 4 white blood, white silk, showing that two pairs of factors interact, viz., C., turning blood yellow, but by itself not affecting silk, and Y, which affects visibly neither blood nor silk unless combined with C, when it makes the silk yellow. Moreover, not all individuals of white blood, white silk strains are double recessives. Some are either homozygous or heterozygous for the dominant yellow silk factor, Y, though they are of course homozygous recessives for the blood color, viz., cc.

If the yellow blood of the silkworm is due to xanthophyl from mulberry leaves, upon which point Dubois ('09) expresses some doubt, there would seem to be involved in this case two recessive decolorizing enzymes, one of general nature, c, turning the blood white, and the other, y, acting specifically through the nuclei of the silk glands to decolorize their product, the glands themselves, as Ude points out, not being affected. Either of the recessive decolorizers must be present in homozygous or duplex quantity in order to produce a visible result, i. e., CcYy would be yellow blood, yellow silk; Ccyy, yellow blood, white silk; ccYY or ccYy, white blood, white silk. This assumption necessitates no change in Ude's formulas and gives a working hypothesis that may be of value regarding the factors.

SUMMARY

1. Numerous (ca. 44) conspicuous blue-green caterpillars appeared in August, 1920, in closely inbred stock of the protectively colored grass-green caterpillars of *Colias philodice*.

2. The three broods in which they first appeared clearly showed the 3:1 ratio, grass-green (i.e., yellow-green) being dominant, blue-green, recessive. Hence their six parents were all heterozygous (grass-green) dominants. Five of the latter were brothers and sisters, one a cousin, derived from two pairs of grandparents.

3. The matings of each of these two pairs of grandparents must have been of the type: normal (dominant) grass-green \times heterozygous grass-green. All four had been hibernating caterpillars, the offspring of a wild female (with white wing color) of the fall brood of 1919. She, or her mate, was probably also a heterozygous grass-green caterpillar, the other member of the pair being normal. The mutation is thus to be traced back to one of the individuals of this pair.

4. This is an example of recessive mendelian factors, (mutations) existing unsuspected in true-breeding wild stock, but brought to light by inbreeding.

5. In the second generation, the offspring of two recessives, blue-green \times blue-green, bred true. A pair of grass-green heterozygotes gave an excess of recessives, but the total numbers were very small.

6. The mutation is not sex-limited.

7. Two non-inherited pigments derived from the chlorophyll of the food plant, and probably only slightly modified by digestion and combination with other proteids (Poulton, '85), are the physical basis of the dominant and recessive colors. They are xanthophyl, a yellow pigment, and chlorophyl *a*, of blue-green color. Both probably exist together in normal grass-green hemolymph.

8. The hereditary nuclear enzyme, or recessive gene, involved in this case is a decolorizer (inhibitor) of xanthophyl. Since it is recessive, it must be present in double dose (homozygous condition) in order to produce its effect. It

probably acts primarily through the nuclei of the intestinal epithelium upon the digesting chlorophyll, and thereby upon the blood itself. It affects directly, or indirectly through the blood, every stage in the life-history of the insect.

9. The egg of the butterfly from a blue-green caterpillar is pure alabaster-white, not the normal cream-white, an effect due to the lack of yellow in the blood of the mother.

10. The blueness of the blood of the larva becomes visible just before the second molt by the stretching of the skin, then relatively thick. After this molt the larva is conspicuously bright blue-green ('light procelain green' of Ridgeway).

11. There are no intermediates. It is a sharply discontinuous variation.

12. The pupa is only slightly less blue than the larva. Its cast cuticula is white, not the normal yellow.

13. The wing color of the adult is unaffected by the mutation, for females from blue-green caterpillars have either yellow or white wing colors, as determined by another independent pair of genes.

14. The eye color of the adult is affected by the mutation, probably through action of the blood. It lacks yellow and is of a bluish green ('mineral green' rather than the normal 'apple green').

15. The determination of the color of the hemolymph is primary, or direct, but that of egg, pupal cuticula and of the eye secondary, or indirect. The following observation favors the idea that at the points last mentioned the action is indirect:

16. Hymenopterous parasites, *Apanteles flaviconchae* Riley, emerging from a grass-green caterpillar spin bright golden-yellow cocoons, those emerging from a blue-green caterpillar spin white cocoons. The lack of xanthophylloid pigment in the blood of the blue-green host, or possibly the action of a yellow-inhibiting enzyme produced by the gene in the blood, changes the color of the secretion of the silk glands of the parasite from yellow to white. Presumably, the blood affects in a similar manner the hypodermis and cuticular secretion of the hypodermal cells of the host.

17. That blueness of blood and hypodermis, whiteness of cuticula, etc., are not due to a purely maternal blood variation but to a typical recessive mutation, is evidenced by the large numbers of blue-green caterpillars that appeared in two broods of the original generation, both showing a typical 1:3 ratio, and by the production in the next of both blue-green and grass-green larvae by heterozygous [grass-green] parents and of blue-green by homozygous [blue-green] parents.

18. Normal grass-green caterpillars of *C. philodice* are protected by their color against the attacks of English sparrows. From an outdoor culture exposed to sparrows for twelve days the conspicuous blue-green caterpillars were practically all eliminated, while many grass-green larvae were left unmolested (as seen by comparison with two covered lots of the same brood).

19. The blue-green caterpillar is as vigorous and disease-resistant as the normal, but the adults are less active and less inclined to mate than their heterozygous, or normal homozygous, brothers and sisters.

20. A discussion of the relation of the blue-green blood pigment to similar pigments in plants and animals, including a mutation in blood pigment in the silkworm, concludes the paper.

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PLATE

PLATE 1

DESCRIPTION OF FIGURES

Photographs by Prof. Leland Griggs and Mr. Kenneth Robes, colored from life by Prof. Adelbert Ames.

1 Blue-green mutant caterpillar of *Colias* (*Eurymus*) *philodice*,—a recessive to yellow-green. Xanthophylloid yellow blood pigment is suppressed by the recessive factor. Note absence of pink lateral line as well as of the yellow component of the green seen in figure 1 a.

1a Normal full-grown caterpillar of *Colias* (*Eurymus*) *philodice*.

2 Blue-green pupa.

2a Normal pupa.

3 Cast cuticula of blue-green pupa.

3a Cast cuticula of normal pupa.

4 Blue-green eye of adult butterfly from a blue-green caterpillar.

4a Normal eye color.

5 White cocoons spun by larvae of *Apanteles flaviconchae*, a parasitic braconid, that had emerged from a blue-green caterpillar. Note the absence of normal yellow pigmentation. $\times 1\frac{1}{2}$.

5a Bright yellow cocoons spun by larvae of the same species of braconid that had fed upon, and emerged from, a normal yellow-green caterpillar. The yellow color is probably due to xanthophylloid pigment in the hemolymph of the host that is suppressed in the blue-green caterpillar.

6 Adult braconid parasite of the caterpillar of *Colias* (*Eurymus*) *philodice*. *Apanteles flaviconchae* Riley. $\times 18$.



Resumen por el autor, Lewis V. Heilbrunn.

Cambios de la viscosidad del protoplasma durante la mitosis.

En estudios precedentes el autor ha observado los cambios de la viscosidad del protoplasma durante la mitosis. En el presente trabajo ha intentado determinar la magnitud de estos cambios y su exacta relación temporal con el proceso de la mitosis. El método de la centrifugación ha sido empleado para medir la viscosidad del protoplasma. La mayor parte de las medidas fueron llevadas a cabo en el óvulo del lamelibranquio Cumingia. Los óvulos fueron centrifugados con cortos intervalos durante el tiempo transcurrido entre la fecundación y segmentación. La velocidad del movimiento granular bajo la influencia de una fuerza centrífuga determinada han sido considerada como la medida de la viscosidad protoplásmica. Tanto durante las divisiones de maduración como en la segmentación, la aparición del huso está precedida por una aumento brusco en la viscosidad, seguido de una brusca disminución de la misma. Al final de la mitosis, inmediatamente antes de dividirse la célula, la viscosidad aumenta marcadamente. Las medidas cuantitativas han sido obtenidas y los resultados han sido representados gráficamente. Durante las divisiones de maduración y la segmentación del óvulo del anélido Nereis tienen lugar cambios de viscosidad semejantes a los de Cumingia.

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PROTOPLASMIC VISCOSITY CHANGES DURING MITOSIS¹

L. V. HEILBRUNN

ONE CHART

THE PROBLEM

Earlier work showed that the segmentation of the sea-urchin egg and the appearance of the mitotic spindle in this egg depend upon a viscosity increase in the cytoplasm (Heilbrunn, '15, '17, '20). During mitosis protoplasmic viscosity changes were demonstrated. It is of interest now to inquire whether similar viscosity changes occur in the dividing eggs of other forms. If the mitotic mechanism depends upon viscosity changes, then such changes should be present in all dividing cells. Furthermore, it has been thought advisable to determine more accurately the magnitude of these changes.

Most of the work was done with the *Cumingia* egg, although some data from the sea-urchin and *Nereis* eggs is included for the sake of comparison. The *Cumingia* egg is immature at fertilization and therefore offers an interesting comparison to the egg of the sea-urchin. In *Cumingia*, three cell divisions occur in a short space of time between fertilization and the first cleavage. Do viscosity changes accompany these divisions and do they show the same time relation to the mitotic process that was previously demonstrated for the sea-urchin egg?

At Woods Hole *Cumingia tellinoides* is fairly common, and during the summer of 1920 good material was generally available throughout July and August. My thanks are due to Prof. F. R. Lillie, director of the Marine Biological Laboratory, for the privilege of carrying on the work there.

¹ Contribution from the Zoölogical Laboratory of the University of Michigan.

THE METHOD

In general the viscosity of a fluid may be determined in any one of three ways. The usual method is to measure the rate of flow of the fluid through a tube. One can also obtain a measure of the viscosity by determining the rate at which solid substances pass through the fluid, or one can measure viscosity by determining the force necessary to push solid materials through the fluid. All three methods are used by physicists, but only the second and third can be applied to the living cell. When the protoplasm is not very viscous and contains large granules of relatively high specific gravity, these granules may be seen to move under the influence of gravity. The time of fall can then be used as a measure of the viscosity of the protoplasm. This method has been applied to some plant cells by Heilbronn ('14). In animal cells generally the granules do not appear to be affected by gravity, and a stronger force is necessary to move them. Such a force can be obtained with the centrifuge, and the centrifugal method of measuring viscosity is doubtless capable of wide application. With the centrifuge both the second and third methods of measuring viscosity can be used. One can either determine the speed attained by protoplasmic granules under the influence of a given centrifugal force or one can determine the amount of force necessary to produce a certain degree of movement. To some extent both methods were used in earlier work.

At this point it might be well to consider the theory of viscosity measurements obtained by observing the movements of solids through liquids. This method has been used comparatively rarely by physicists and chemists. Arndt ('07) used it to determine the viscosity of fused salts. A general discussion of the subject with references to literature is given by Ladenburg ('07). When fairly small particles are made to move through viscous liquids, this movement is found to occur at a uniform rate. For such a movement in the case of spherical particles, Stokes ('50) derived a formula often used by physicists. In a suitable form it is:

$$V = \frac{2g(\sigma - \rho)a^2}{9\mu}$$

in which V is the velocity of movement, g the gravity constant, σ the specific gravity of the particle, ρ the specific gravity of the liquid, a the radius of the particle, and μ the viscosity of the liquid.

In the mathematical derivation of Stokes' formula various assumptions are made. Arnold ('11) has considered the significance of all these assumptions. He has shown that for small particles dropping through viscous liquids, Stokes' law holds. When the particles drop through comparatively narrow tubes of the liquid, then a correction must be made for the effect of the walls of the tube. For comparative tests of viscosity in which the same tube is used throughout, this correction disappears.

Stokes' law applies to the action of gravity. In order to make it apply to centrifugal force, one must insert a factor c for the centrifugal force in terms of gravity. The formula then becomes:

$$V = \frac{2cg(\sigma - \rho)a^2}{9\mu}$$

Centrifugal force may be many times gravity. It might be thought that with such a powerful force, the velocity of the particles might become so great that Stokes' law would no longer hold. In the present experiments no such high velocity was ever attained. The speed of the granules ranged approximately from 0.0002 to 0.0018 cm. per second. This is a very low rate of speed.

We are reasonably safe, therefore, in assuming that Stokes' law can be applied to the movement of cytoplasmic granules under the influence of centrifugal force. With a given centrifugal force, the speed with which the granules move is accordingly inversely proportional to the viscosity of the fluid through which they move, the cytoplasm. But this speed also depends on two other factors, the radius of the particle, and the difference in specific gravity between the particle and the fluid of the cytoplasm. If the simple formula is to be used to measure viscosity

it is important that these factors remain constant. During mitosis in the *Cumingia* egg the granules do not change appreciably in size. This constancy is shown in the drawings of Morgan ('10).² Perhaps the specific gravity of the granules changes. In the egg there are two sets of granules, one set lighter, the other heavier than the cytoplasm. When the eggs are centrifuged, the lighter granules go to one pole, the heavier granules to the opposite pole. It has been found that whenever the lighter granules move more rapidly, the heavier granules also move more rapidly, and a drop in the speed of the lighter granules is always accompanied by a drop in the speed of the heavier granules. If the change in speed were in any case due to a change in specific gravity of the granules, we would have to assume that an increase in the specific gravity of the lighter granules was in every case accompanied by a corresponding decrease in the specific gravity of the heavier granules, and vice versa. At first sight this appears impossible, but it is not. An entrance of water into the granules would increase the specific gravity of the lighter granules and decrease the specific gravity of the heavier granules, and an exit of water would have the opposite effect. But such a passage of water would of necessity produce very decided changes in the diameter of the granules and these changes, as we have already noted, do not occur.

Since, therefore, the diameter of the granules and their specific gravity can be taken as constant, the velocity of the granules affords a measure of the viscosity of the cytoplasm. In measuring changes in protoplasmic viscosity, one can either determine the variation in the speed of the granules with a certain amount of centrifugal force or one can determine the amount of force necessary to produce a certain velocity of the granules. In actual practice it is easier to use the first method, especially if a hand centrifuge is employed. With such a centrifuge one acquires the knack of turning very regularly at a given speed but it is somewhat more difficult to change from one speed to another.

² It was also shown by careful measurement of granules in living eggs.

In all of the measurements made in 1920 a new model Bausch & Lomb centrifuge with haematocrit attachment was used. With this instrument one turn of the high-speed handle produces 130 turns of the tubes. The distance between the ends of the tubes is about 14.6 cm. The centrifugal force in dynes is given by the formula of Huyghens:

$$C = 4\pi^2 n^2 r m.$$

in which C is the centrifugal force, n is the number of turns per second, r is the radius of the circle described by the ends of the tubes, and m the mass. The force of gravity is 980.36 dynes (at Boston) times m . In order to express the centrifugal force in terms of gravity, we must therefore divide by 980.36 m . Thus:

$$C' = \frac{4\pi^2 n^2 r m}{g m} = \frac{4\pi^2 n^2 r}{g}$$

in which C' is the centrifugal force in terms of gravity, and g is the gravity constant. With the hand centrifuge it was found most convenient to maintain a speed of one turn of the high-speed handle per second. This represents a force 4968 times gravity.

When the *Cumingia* egg is centrifuged, the oil globules pass to one pole, the yolk and pigment granules to the opposite pole. The egg then appears banded and three zones show, a dark zone contains the oil globules, a pigmented zone contains the yolk granules and the pink pigment, and between the two is a clear and transparent zone. In referring to these zones they will be spoken of as the dark zone, the pigment zone, and the hyaline zone. The appearance of these zones is beautifully shown in the illustrations of Morgan.

THE VISCOSITY CHANGES IN THE CUMINGIA EGG

The protoplasm of the unfertilized *Cumingia* egg has a relatively low viscosity. A number of tests were made during the season. At a speed of one turn per second of the high-speed

handle of the centrifuge, usually two or three seconds elapsed before the first indication of zones appeared. In one case four seconds were required, but this was exceptional. In another case a single turn was sufficient to show zones, but these eggs of unusually low viscosity were not absolutely normal and at segmentation they showed poorly indented cleavage furrows. Apparently the viscosity varies with the temperature, decreasing slightly as the temperature rises from 19° to 25°. More data are necessary, however, in order to establish this point.

The viscosity changes during mitosis are shown in the following tables. Eggs were centrifuged at rapid intervals between fertilization and cleavage. One series of tests was made in which the centrifuge handle was turned three times in three seconds, another series in which it was turned four times in four seconds, and so on. In all of the tests recorded here, a uniform speed of one turn per second was maintained. This was continued for from two to twenty seconds in the various tests. Because of the difficulty in starting, it is not easy to turn the handle twice in exactly two seconds. After a turn or two has been made, however, it is a simple matter to keep the speed uniform.

In the tables the first column shows the number of seconds elapsed after fertilization at the time a given test was made. In working with various sets of eggs on different days, the length of time between fertilization and cleavage varies considerably. In order to compare one set of eggs with another, it is necessary to reduce all the time records to some standard basis. The average time between fertilization and cleavage is about fifty minutes. Fifty minutes was therefore taken as a standard time interval, and by a simple calculation it was determined in each case how much time would have elapsed if the total time until cleavage had been fifty minutes. These figures are given in the second column and are referred to as 'standard time.' There is always some variation in the time of cleavage of individual eggs of any one batch. The time of first cleavage was taken as the time when 50 per cent of the eggs had completely divided. In order to obtain this time, rapid counts had to be made at frequent intervals. These counts are recorded in the third

column in the form of fractions. The numerator represents the number of eggs completely segmented, the denominator the total number of eggs counted.

In the third column also are recorded the observations made during the progress of the tests. It must be pointed out that these observations were necessarily very hasty, and are therefore not as accurate as they might have been if the observer had had time to proceed leisurely. Usually the tests were made at very frequent intervals, and all observations had to be completed in the shortest possible time.

TABLE 1
Three-second tests. Temperature, 24°

ACTUAL TIME AFTER FERTILIZATION	STANDARD TIME	OBSERVATIONS
<i>minutes</i>	<i>minutes</i>	
5	4.9	Dark zone distinct in some eggs
7½	7.3	3 zones appear in some eggs; others lack zones, these have polar body.
10	9.7	No zones visible. All eggs have polar body.
12½	12.1	No zones.
15	14.5	No zones.
17½	17.0	A few eggs show zones (perhaps 10 to 20 per cent). Most eggs lack zones.
20	19.4	Dark zone present, often it is not very sharply defined.
22½	21.8	No zones.
25	24.3	No zones.
27½	26.7	No zones.
30	29.1	No zones. The two pronuclei appear large, but they are not yet apposed.
32½	31.6	No zones.
35	34.0	No zones. The pronuclei are apposed.
37½	36.4	No zones. The pronuclei have fused.
40	38.8	Most eggs lack zones. A few show the dark zone, but it is not very sharply marked off.
43	41.7	Eggs generally lack zones. 1 or 2 perhaps show dark zone.
46	44.7	No zones.
48½	47.1	No zones. Many eggs in mitotic elongation stage. Some are beginning to segment.
50-51		12/100 segmented.
51½		23/50 segmented. This taken as the time of segmentation.
52½		37/50 segmented.

TABLE 2

Four-second tests. Eggs fertilized at 4.30 p.m. Temperature at 4.29 p.m., 25.6°; at 5.15 p.m., 25.8°

ACTUAL TIME AFTER FERTILIZATION	STANDARD TIME	OBSERVATIONS
<i>minutes</i>	<i>minutes</i>	
2	2.4	Three zones show plainly.
4	4.8	In some eggs zones appear faintly. Most eggs lack zones. A few polar bodies observed (5 min. after fertilization).
6	7.2	No zones. Polar bodies generally present.
8	9.6	Most eggs lack zones. A few eggs have zones indicated.
10	12.0	Three zones show plainly.
12	14.5	Zones indicated in many eggs, never plain..
14	16.9	Zones indicated in many eggs, never plain.
16	19.3	No zones generally (a few eggs have zones faintly indicated).
18	21.7	No zones generally. The second polar body is forming (or has formed).
20	24.1	No zones.
22	26.5	No zones.
24	28.9	No zones.
26	31.3	No zones. Pronuclei large, not apposed.
28	33.7	Zones show faintly in some eggs. Pronuclei large, apposed.
30	36.1	Zones appear; usually they are not very sharply marked off. Pronuclei large, apposed.
32	38.6	Zones show fairly plainly. Pronuclei usually broken down.
34	41.0	Zones show fairly plainly.
36	43.4	Zones usually show fairly plainly. In some eggs they appear indistinct.
38	45.8	Some eggs show zones. Most eggs lack zones. A few eggs are in the mitotic elongation stage.
40	48.2	No zones generally. Eggs have elongated and some have begun to segment.
41 $\frac{1}{4}$		8/25 segmented.
41 $\frac{3}{4}$		15/25 segmented. 41 $\frac{1}{2}$ minutes taken as the time of segmentation.
42 $\frac{1}{2}$		21/25 segmented.

TABLE 3

Five-second tests. Eggs fertilized at 12.15 p.m. Temperature at 12.18 p.m., 22.0°; at 1.10 p.m., 23.6°

ACTUAL TIME AFTER FERTILIZATION	STANDARD TIME	OBSERVATIONS
<i>minutes</i>	<i>minutes</i>	
2½	2.2	Three zones show plainly.
5½	4.8	Most eggs lack zones. Some eggs show them.
8	7.0	Eggs generally lack zones.
11½	10.1	No zones.
14	12.3	No zones.
16½	14.5	No zones.
18½	16.2	No zones.
21	18.4	Zones appear in many eggs, others lack them.
23½	20.6	Zones appear generally.
26	22.8	Zones in some eggs, others lack them.
28½	25.0	No zones.
30½	26.8	No zones.
33	29.0	No zones.
36	31.6	No zones.
39½	34.6	No zones. Pronuclei large.
42½	37.3	No zones show clearly. Some show zones indistinctly.
45	39.5	Eggs generally show zones.
47½	41.9	Eggs generally show zones.
50½	44.3	No zones.
53	46.5	No zones. Eggs generally in mitotic elongation stage.
56		13/40 segmented.
58		21/31 segmented. 57 minutes taken as the time of segmentation.
59		83/100 segmented.

TABLE 4

Six second tests. Eggs fertilized at 11.00 a.m. Temperature at 10.59 a.m., 23.4°; at 11.52 a.m., 26.4°

ACTUAL TIME AFTER FERTILIZATION	STANDARD TIME	OBSERVATIONS
<i>minutes</i>	<i>minutes</i>	
2½	2.6	Three zones show plainly.
5	5.3	Zones when present are indistinct and not sharply marked off.
7½	7.9	No zones. Eggs have polar bodies.
10	10.5	No zones.
12	12.6	Zones indicated. The dark zone is often plain but the pigment zone does not show.
14	14.7	Zones show generally. The pigment zone is not always distinct.
16	16.8	Three zones show plainly.
18	18.9	Three zones show plainly.
20	21.1	Dark zone sharply defined. Pigment zone usually not sharply defined.
22	23.2	No zones.
24	25.3	No zones.
26	27.4	No zones generally (a few exceptions).
28	29.5	No zones generally (a few exceptions).
30	31.6	No zones.
32½	34.2	No zones.
35	36.8	Zones appear. They are not very sharply defined.
37	38.9	Three zones show plainly.
39	41.1	Three zones show plainly.
41	43.2	Three zones show plainly.
43	45.3	Many eggs show zones; in these the zones are not as sharply defined as in last test. Some eggs lack zones.
45	47.4	Some eggs with zones. Most eggs in mitotic elongation stage or with cleavage furrow beginning. These lack zones.
47¼		7/25 segmented.
47¾		13/25 segmented. 47½ minutes taken as the time of segmentation.
49		19/25 segmented.
50		23/25 segmented.

TABLE 5

*Seven-second tests. Eggs fertilized at 5.20 p.m. Temperature at 5.19 p.m., 22.3°;
at 6.17 p.m., 24.°*

ACTUAL TIME AFTER FERTILIZATION	STANDARD TIME	OBSERVATIONS
<i>minutes</i>	<i>minutes</i>	
2	1.9	Three zones show plainly.
4	3.7	Three zones show, but not as plainly as in previous test.
6	5.6	Most eggs show three zones. In some eggs these zones are indistinct.
8	7.4	Generally no zones. Polar body beginning to appear in one egg.
10	9.3	No zones, polar bodies general.
12	11.1	No zones.
14	13.0	No zones (perhaps they are indicated in a few eggs).
16	14.8	Many eggs show zones; these are not very distinct.
18	16.7	Three zones show plainly in most eggs.
20	18.5	Three zones show plainly.
22	20.4	Three zones show plainly.
24	22.2	Some eggs show zones, others lack zones or show them indistinctly.
26	24.1	No zones generally. Many eggs are elongated, indicating mitotic elongation for second maturation division.
28	25.9	No zones generally (a few exceptions).
30	27.8	No zones generally (a few exceptions).
32	29.6	No zones generally.
34	31.5	No zones generally. Pronuclei fairly large, not apposed.
36	33.3	No zones generally. Pronuclei large, apposed or nearly apposed.
38	35.2	Zones appear indistinctly in most eggs; in a few eggs they are distinct. Pronuclei large, apposed.
40	37.0	The three zones show quite distinctly. Pronuclei are still present.
42	38.9	Three zones show plainly. Pronuclei have broken down in most eggs.
44	40.7	Three zones show plainly.
46	42.6	Three zones show plainly, they appear sharper and clearer than at any previous test.
48	44.4	In most eggs, three zones distinct; in some, no zones.
50	46.3	A few eggs show three zones, the majority lack zones or show them indistinctly. Mitotic elongation is beginning in some eggs.
52	48.1	No zones. Eggs generally show mitotic elongation.
53½		3/25 segmented.
54		10/25 segmented. This taken as time of segmentation.
54½		19/25 segmented.
55		23/25 segmented.

TABLE 6
Eight-second tests. Temperature, 24.2°

ACTUAL TIME AFTER FERTILIZATION	STANDARD TIME	OBSERVATIONS
<i>minutes</i>	<i>minutes</i>	
2	1.8	Zones show.
5	4.6	Dark zone shows, but pigment zone does not.
7½	6.9	No zones. Polar bodies just beginning to be lifted off.
10	9.2	No zones.
12	11.0	No zones.
14	12.8	Most eggs lack zones, a very few show dark zone.
16	14.7	Three zones show plainly.
18	16.5	Three zones show plainly.
20	18.3	Dark zone plain, pigment zone not so distinct.
22½	20.6	Most eggs with zones not very distinct. A few eggs lack zones completely.
26	23.9	No zones.
28	25.7	No zones.
30	27.5	No zones.
32½	29.8	No zones.
35	32.1	No zones. Pronuclei large.
37½	34.4	No zones. Pronuclei large and close to each other.
40	36.7	In many eggs zones show quite distinctly. These apparently have pronuclei broken down. Some eggs lack zones.
42¾	39.2	Three zones show plainly.
44¾	41.1	Three zones show plainly.
47½	43.6	Three zones show plainly.
50½	46.3	No zones.
53½		3/50 segmented.
53¾		7/50 segmented.
54½		9/50 segmented.
54¾		14/25 segmented. 54½ minutes taken as the time of segmentation.
55½		21/25 segmented.

TABLE 7

Ten-second tests. Eggs fertilized at 6.45 p. m. Temperature at 6.57 p.m., 27.4°; at 7.28 p.m., 27.4°

ACTUAL TIME AFTER FERTILIZATION	STANDARD TIME	OBSERVATIONS
<i>minutes</i>	<i>minutes</i>	
2	2.5	Three zones show plainly.
4	4.9	In most eggs three zones show plainly, in some they are not so plain.
7	8.6	Most eggs lack zones, these have first polar body. A few with zones lack polar body.
10	12.3	Three zones show plainly in most eggs.
13	16.0	Three zones show plainly.
15	18.5	Three zones show plainly in some eggs. Other eggs lack zones.
17½	21.6	Zones are indicated, but are not sharply defined.
20½	25.3	No zones.
23	28.4	No zones.
25½	31.4	No zones.
28	34.5	Most eggs lack zones, a few show them. Pronuclei large.
30½	37.7	Three zones show plainly.
32½	40.1	Three zones show plainly.
34½	42.6	Three zones show plainly.
37	45.7	Three zones show plainly in many eggs. Some eggs are in mitotic elongation stage and these lack zones.
39	48.1	No zones. Segmentation beginning in some.
40½		10/25 segmented. This taken as the time of segmentation.
41		17/25 segmented.
41½		22/25 segmented.
42½		23/25 segmented.

TABLE 8

Twelve-second tests. Eggs fertilized at 3.55 p.m. Temperature at 3.51 p.m., 25.6°

ACTUAL TIME AFTER FERTILIZATION	STANDARD TIME	OBSERVATIONS
<i>minutes</i>	<i>minutes</i>	
2	2.2	Three zones show plainly.
5	5.6	Some eggs with zones distinct, others with dark zone fairly sharp, but with pigment zone not well marked off.
8 $\frac{1}{2}$	9.6	No zones. Polar bodies generally present.
11 $\frac{1}{2}$	12.9	Three zones generally present. Pigment zone not always sharply defined.
14	15.7	Three zones show plainly.
16 $\frac{1}{2}$	18.5	Three zones show plainly.
19	21.3	Three zones show plainly in some eggs. In others only the dark zone is clearly defined.
24	27.0	No zones (a few exceptions show indications of a dark zone).
26 $\frac{1}{2}$	29.8	No zones.
29	32.6	Most eggs without zones. Zones are indicated in a few. Pronuclei large.
31 $\frac{1}{2}$	35.4	Zones appear, but they are not very sharply delimited. The pronuclei are large and apposed.
34	38.2	Three zones show plainly.
36 $\frac{1}{2}$	41.0	Three zones show plainly.
38 $\frac{1}{2}$	43.3	Three zones show plainly.
41	46.1	Majority lack zones. Some have zones indicated.
43	48.3	No zones. Cleavage furrows beginning.
44 $\frac{1}{2}$		10/25 segmented. This taken as the time of segmentation.
45		20/25 segmented.
45 $\frac{1}{2}$		23/25 segmented.
45 $\frac{3}{4}$		25/25 segmented.

TABLE 9
Fourteen-second tests. Eggs fertilized at 10.50 a.m. Temperature at 10.49 a.m., 19.9°; at 12.06 p.m., 22.8°

ACTUAL TIME AFTER FERTILIZATION	STANDARD TIME	OBSERVATIONS
<i>minutes</i>	<i>minutes</i>	
2	1.4	Three zones show plainly.
4½	3.2	Three zones show plainly (in some they are not as sharply defined as in previous test).
7	4.9	Three zones show generally, but the pigment zone is not sharply marked off.
10	7.0	Zones are either not present or if present they are indicated rather than sharply defined.
13	9.1	No zones. Polar bodies generally present.
15½	10.9	No zones.
18	12.7	Zones indicated in some eggs.
20½	14.4	Three zones appear plainly in some eggs. In others only the dark zone appears.
23	16.2	Three zones show plainly.
25½	18.0	Three zones show plainly.
28	19.7	Three zones show plainly.
30½	21.5	Three zones show plainly in most eggs, in some they are not sharply defined.
33	23.3	Most eggs lack zones, a few show them. Eggs have one, but not two polar bodies.
35	24.6	No zones. A few eggs were examined carefully and showed second polar body forming.
38	26.8	Zones appear in many eggs. They are not sharply defined.
40½	28.5	Zones do not show plainly. Sometimes the dark zone appears, but the pigment zone does not.
44	31.0	No zones.
46½	32.8	No zones.
49	34.5	Zones indicated, never distinct.
51½	36.3	Zones appear, they are not very distinct. The pronuclei are very large.
54	38.0	Three zones show plainly.
56½	39.8	Three zones show plainly.
59	41.5	Three zones show plainly.
61½	43.3	Three zones generally show plainly (a few exceptions in which zones were not very sharply marked off).
63½	44.7	Three zones generally show plainly (a few exceptions in which zones were not very sharply marked off).
66	46.5	A few eggs show zones plainly. Most eggs are in mitotic elongation stage and lack zones.
68½	48.2	No zones. Eggs generally in mitotic elongation stage.
70		3/25 segmented.
70½		7/25 segmented.
71		15/25 segmented. This taken as the time of segmentation.
71½		19/25 segmented.
72½		22/25 segmented.
73½		23/25 segmented.

TABLE 10

Sixteen-second tests. Eggs fertilized at 11.20 a.m. Temperature at 11.18 a.m., 19.0°; at 12.34 p.m., 23.4°

ACTUAL TIME AFTER FERTILIZATION	STANDARD TIME	OBSERVATIONS
<i>minutes</i>	<i>minutes</i>	
2	1.5	Three zones show plainly.
7½	5.5	Three zones show plainly in most eggs. A few exceptions.
10	7.4	Zones show, but are generally indistinct.
12½	9.2	Zones not distinct. Dark zone indicated, no pigment zone.
15½	11.4	Zones show indistinctly.
18	13.2	Three zones show plainly.
20	14.7	Three zones show plainly.
22½	16.5	Three zones show plainly.
25	18.4	Three zones show plainly.
27½	20.2	Three zones show plainly.
30	22.1	Zones appear, but are not as sharply marked off as in previous test.
33	24.3	Elongated eggs (i.e., those giving off second polar body) lack zones or have them barely indicated. Spherical eggs show zones.
36½	26.8	Zones are indicated, but do not show plainly. The dark zone is more distinct than the pigment zone.
39	28.7	Zones not distinct, but often they can be made out. The dark zone is more plainly indicated than the pigment zone.
41½	30.5	Zones indicated, especially the dark zone.
44½	32.7	No zones generally, a few exceptions.
47½	34.9	Zones indicated generally. Pronuclei large.
50	36.8	Three zones appear distinctly, although they are often not very sharply marked off. Pronuclei large.
52½	38.6	Three zones show plainly. Pronuclei large, their boundaries fading.
55	40.5	Three zones show plainly. Pronuclei not large or prominent; they have probably broken down.
57½	42.3	Three zones show plainly.
60	44.1	Three zones show plainly.
62½	46.0	Some eggs show zones. Many eggs in mitotic elongation stage and lack zones.
65	47.8	Most eggs in mitotic elongation stage or beginning segmentation. They lack zones.
67		5/25 segmented.
67½		8/25 segmented.
67¾		13/25 segmented. 68 min. taken as time of segmentation.
68½		18/25 segmented.
69		20/25
70		22/25
71		23/25

TABLE 11

Eighteen-second tests. Eggs fertilized at 12 m. Temperature at 12.07 p.m., 23.0°; at 1.00 p.m., 24.5°

ACTUAL TIME AFTER FERTILIZATION	STANDARD TIME	OBSERVATIONS
<i>minutes</i>	<i>minutes</i>	
2	1.7	Zones show very plainly.
5	4.2	Three zones show very plainly.
8	6.7	Three zones show very plainly.
11	9.2	Three zones show plainly, but eggs with polar body have hyaline zone clouded over with granules.
14	11.8	Three zones show plainly, hyaline zone clouded with granules.
17	14.3	Three zones show plainly.
19½	16.4	Three zones show plainly.
22	18.5	Three zones show plainly.
24½	20.6	Three zones show plainly. In some eggs hyaline zone is clouded with granules.
27	22.7	Three zones show plainly. In some (elongated eggs), hyaline zone is somewhat cloudy.
30	25.2	Three zones show plainly. A few with hyaline zone cloudy.
33	27.7	Three zones show. Hyaline zone usually cloudy.
36	30.3	Three zones show. Hyaline zone clouded with granules. Pronuclei fairly large.
39	32.8	Three zones show. Hyaline zone somewhat clouded. Pronuclei large, apposed.
42	35.3	Three zones show plainly, hyaline zone not clouded. Pronuclei large, apposed.
45	37.8	Three zones show plainly. In some eggs pronuclei are present, in others they have disappeared.
48	40.3	Three zones show plainly. In most eggs pronuclei have disappeared.
50½	42.4	Three zones show plainly.
53	44.5	Three zones show plainly in most eggs. A few are in mitotic elongation stage and have zones indicated.
55½	46.6	Elongated eggs and dividing eggs have zones indicated. They are not distinct, but can be made out.
59		7/25 segmented.
59½		10/25 segmented. This taken as the time of segmentation.
60		18/25 segmented.
62		17/25 segmented. Thus only about 70 per cent segmented.

TABLE 12

Eighteen-second tests. Eggs fertilized at 11.40 a.m. Temperature at 11.42 a.m., 24.3°; at 12.30 a.m., 24.0°

ACTUAL TIME AFTER FERTILIZATION	STANDARD TIME	OBSERVATIONS
<i>minutes</i>	<i>minutes</i>	
3	3.1	Three zones show plainly.
6	6.2	Many eggs show three zones plainly. In some eggs hyaline zone is clouded with granules and sometimes the pigment zone is not sharply marked off.
9	9.3	Dark zone and pigment zone show plainly, hyaline zone clouded somewhat.
14	14.4	Three zones show plainly.
22	22.7	Zones appear, but are not always sharply marked off.
25	25.8	Dark zone sharply defined, pigment zone not sharply marked off.
29	29.9	Zones do not show distinctly. They are indicated rather than sharply marked off. Dark zone plainer than pigment zone.
32½	33.5	Zones show more plainly than in previous test. Pronuclei apposed, apparently fused.
36	37.1	Three zones show plainly.
39	40.2	Three zones show plainly.
41	42.3	Three zones show plainly.
43	44.3	In most eggs, three zones show plainly. A few eggs are elongated and in these there is no distinct pigment zone; the dark zone appears, but is not well marked off.
45½	47.2	Most of the eggs are elongated. These show no zones. Other eggs show zones.
48½		24/50 segmented. This taken as the time of segmentation.
49½		43/50 segmented.

The tables show that the viscosity of the protoplasm undergoes marked changes. With the same centrifugal speed sometimes two seconds are sufficient to produce zones, sometimes sixteen seconds must elapse before zones appear. In the latter case obviously the velocity of the granules is only one-eighth as great. Since the viscosity varies inversely as the velocity (see previous discussion) this indicates an eight-fold increase in viscosity. The number of seconds necessary to cause the appearance of zones is thus an inverse measure of the velocity of granular

movement and a direct measure of the viscosity. As shown previously it requires on the average two turns of the centrifuge handle (in two seconds) to produce zones in unfertilized eggs. An arbitrary scale of viscosity may be established and the viscosity of the protoplasm of unfertilized eggs can be taken as 2. At a stage when sixteen turns are required to show zones the viscosity in terms of the arbitrary unit is 16.

From the data in the tables a viscosity curve may be plotted. This is not, however, a simple matter. In general it is not possible to obtain direct readings of viscosity. Usually each test indicates that the viscosity of the protoplasm is above or below a certain value, but it does not show how much above or below. Moreover, there is often some variation in the eggs of a given lot, especially at times of transition. By noting for each test whether the viscosity is above or below a certain value a curve may be plotted by drawing a line above the one set of points and below the other set.

The following symbols were used in marking out points:

∨ = Zones show plainly in all eggs. Viscosity above value at apex of *V*.

∧ = No zones appear in any eggs. Viscosity below value at apex of inverted *V*.

○ = Zones appear but indistinctly (usually dark zone plainer than pigment zone). Viscosity about equal to value at center of circle.

The above three symbols were combined in a variety of ways.

× = Eggs with zones and without zones about equal in number.

× = Most eggs with zones, a few without.

× = Most eggs without zones, a few with.

In those cases where zones appeared indistinctly, the arc of a circle was added to the upper or lower portion of any of the last three symbols.

In the curve the abscissa represents time in minutes after fertilization. Of course for purposes of comparison the so-called 'standard time' had to be employed. The ordinates represent comparative viscosity values on the basis of the arbitrary scale mentioned above.

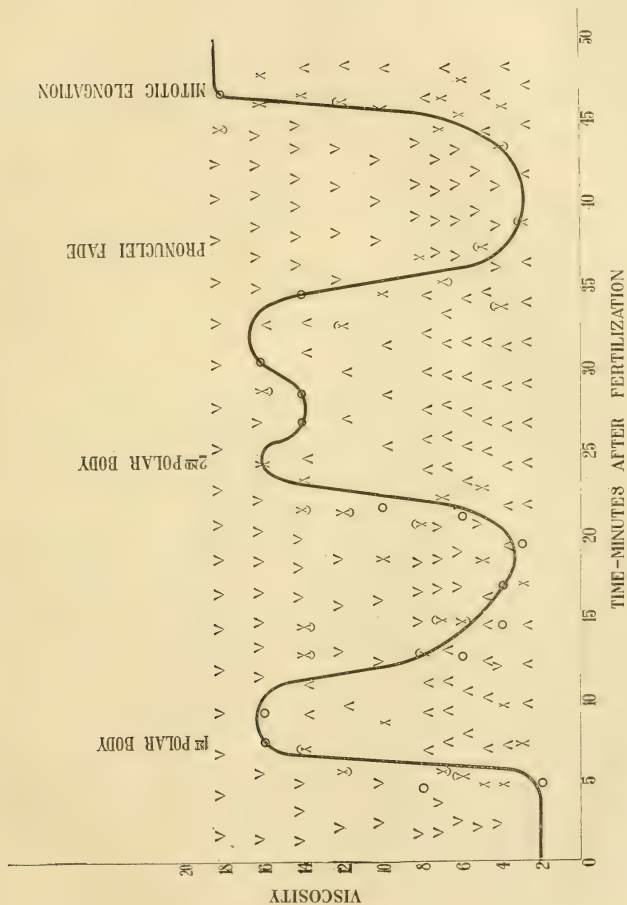
Not all of the available data were included in the curve. All of the tests in table 12 were omitted. Introduction of these figures would not have changed the curve. One test is included which is not given in the tables. This is a twenty-second test made when 5 per cent of the eggs had segmented, hence about a minute before 50 per cent had segmented.

Curve and tables show that there are three times when the viscosity is high. These are just before the first polar body is given off, just before the second polar body is given off, and just before the completion of the first cleavage division. Thus in every case immediately before the division of the cell, there is an increase in viscosity. There is also a high viscosity in the early stages of each mitosis. Generally the completion of one division is bound up with the preparation for the next. As the first polar body is given off, the second maturation spindle begins to form,³ and the viscosity increase at this time is probably causally related to the appearance of the spindle. As the second polar body is given off there is again a viscosity increase. After the completion of the second maturation division the viscosity decreases slightly. It soon rises again in preparation for the cleavage spindle. As the cleavage spindle forms the viscosity drops and remains low until immediately before cleavage, when there is a sharp increase. In general the viscosity increase at the conclusion of each mitosis is directly related to the appearance of the spindle in the mitosis which follows. The concluding phases of one division are bound up with the initial phases of the next. The same sort of phenomenon was previously found to occur in successive cleavages in the sea-urchin egg.

COMPARISONS WITH OTHER EGGS

One point emphasized by the curve is the sudden increase in viscosity just before division. This increase preceded division by a very short interval. The curve shows the interval to be about two minutes or a trifle more. It must be remembered,

³ The second maturation spindle is a new structure and does not result from a transformation of the first maturation spindle. This is shown by the observations of Griffin ('99), Lillie ('01), and various other observers.



however, that the time of cleavage is taken as the time of completed segmentation. The interval between viscosity increase and the first constriction of the cell is somewhat less. Obviously it is not much more than a minute.

In the sea-urchin egg a similar viscosity increase occurs just prior to division. This was indicated by some of my earlier data (cf., for example, the second table on page 216 of my 1920 paper). In earlier experiments, however, I was primarily interested in the viscosity increase which precedes the appearance of the spindle, and I did not study carefully the viscosity at the conclusion of mitosis. The intervals between tests were too large and, moreover, I took as the time of segmentation the time when the first eggs began to divide. It is much more exact to consider as the time of segmentation the moment when 50 per cent of the eggs have divided.

In *Arbacia* there is a rise in viscosity before division. This is shown conclusively by some tests made in 1919. In these tests an old-model Bausch & Lomb centrifuge was used. It differs from the new model in having a shorter distance between the ends of the tubes, the radius of turn being 6 cm.

June 30, 1919. Temperature 18.0° at 10.58 A.M., 19.0° at 11.42 A.M. *Arbacia* eggs were fertilized at five-minute intervals. In finger-bowl *A* eggs were fertilized at 10.55 A.M., in *B* at 11.00 A.M., and in *C* at 11.05 A.M. At 12.10 $\frac{1}{2}$ P.M. less than 1 per cent of the eggs in *A* had begun segmentation. At 12.10 $\frac{3}{4}$ P.M. eggs from *A* and *B* were centrifuged simultaneously in separate tubes, the high-speed handle being turned 30 times in 25 seconds. On examination of centrifuged eggs, the great majority of eggs from *A* showed no hyaline zone, only about 10 to 20 per cent showing such a zone. The centrifuged eggs from *B* all showed a hyaline zone plainly extending one-third of the distance through the egg.

At 12.17 $\frac{1}{4}$ P.M. about 5 per cent of the eggs in *B* were beginning to segment. Starting at 12.17 $\frac{1}{2}$ P.M., eggs from *B* and *C* were centrifuged simultaneously, the high-speed handle being turned 30 times in 28 seconds. When eggs from *B* were examined, there was no hyaline zone in any of the eggs. Eggs from *C* all showed a hyaline zone, sometimes indistinct, sometimes extending a third of the distance through the egg.

Other tests gave similar results. Evidently just before division in *Arbacia* as in *Cumingia* there is a sudden increase in

viscosity. This increase is of considerable importance and can of course be studied experimentally in the same manner that the viscosity increase in early prophase has been studied. This I propose to do in the near future.

The viscosity changes in *Arbacia* and *Cumingia* are absolutely parallel. In each case the appearance of a spindle is preceded by an increase in viscosity and followed by a decrease in viscosity. And in both eggs division of the cell is immediately preceded by a viscosity increase.

Besides the two eggs already mentioned the egg of *Nereis* was also studied. The *Heteronereis* form of *Nereis limbata* is common at Woods Hole. Its early development is well known through the studies of E. B. Wilson and of F. R. Lillie. The egg is immature at fertilization and three complete mitoses occur between fertilization and cleavage. In *Nereis* viscosity changes are not as sharply marked as in *Cumingia* or *Arbacia* and the egg is therefore more difficult to study. The *Nereis* egg is much larger than the eggs of the other two forms studied and it contains a considerable amount of yolk material. In proportion to the size of the egg, the spindle is small. We might expect that the viscosity changes associated with the appearance of the spindle would be masked in an egg when the spindle constitutes but a small fraction of the material of the egg.

A number of tests were made on the *Nereis* egg, but only one series is included here, these are given in table 13. All tests were made at a uniform centrifugal speed. The new-model Bausch & Lomb centrifuge was used and the handle was turned five times in five seconds.

The table shows that in the immature *Nereis* egg the protoplasm is very stiff. Soon after fertilization and the disappearance of the germinal vesicle the viscosity decreases. Following this there are four times of viscosity increase, at 20, 35, 47, and 65 minutes after fertilization. These viscosity increases presumably correspond with, 1) early prophase of first maturation division; 2) conclusion of first and early prophase of second maturation division; 3) conclusion of second maturation division and early prophase of cleavage mitosis; 4) conclusion of

TABLE 13

Five-second tests. Nereis. Eggs fertilized at 10.55 a.m. Temperature at 11.11 a. m., 23.7°; at 12.20 p.m. 24.9°

TIME AFTER FERTILIZATION	OBSERVATIONS
2	No hyaline zone. Germinal vesicle present.
5	No hyaline zone. Germinal vesicle present.
8	No hyaline zone. Germinal vesicle nearly faded out.
11	Hyaline zone beginning to appear. No germinal vesicle.
14	Hyaline zone appears plainly; it extends through about one-fifth of egg.
17	Hyaline zone not pronounced. In some eggs it is absent; in others, if present, it is indicated rather than shown plainly.
20	Hyaline zone is absent generally (perhaps it is indicated in a few).
23	Hyaline zone plain. About one-fifth.
26	Hyaline zone appears plainly.
29	Hyaline zone can be seen, but in most cases it is indistinct.
32	Hyaline zone can be seen, it is generally narrow and not distinct.
35	Generally no hyaline zone.
38	Hyaline zone present. It is generally narrow, but distinct and clear.
41	Hyaline zone plain, extending through one-fifth of egg.
44	Hyaline zone narrow if present at all.
47	No hyaline zone.
50	No hyaline zone.
53	No hyaline zone.
56	In many eggs hyaline zone is wide, clear, plain. It extends through one-fourth of egg, and its inner margin is almost at the equator.
62	Hyaline zone wide and clear as in previous test.
65	No hyaline zone in any eggs.
67	Segmentation beginning generally.
67½	5/50 segmented completely.
68	8/25 segmented completely.
68½	17/25 segmented completely.
69	21/25 segmented completely.
70	49/50 segmented completely.

cleavage division.⁴ Of the first three periods of increased viscosity the third, as might be expected, is the longest. It corresponds to the second period of increased viscosity in the *Cumingia* egg.

⁴According to Wilson ('92), the first polar body is formed thirty-five to forty minutes after fertilization, the second polar body ten to twelve minutes later, and the egg divides after about seventy-five minutes. Evidently Wilson worked at about the temperature of the above experiment. At lower temperatures the times are of course longer.

DISCUSSION

In the last few years the importance of protoplasmic viscosity changes has been increasingly realized. Of all the properties of protoplasm the fact that it is a viscous fluid is perhaps its most general characteristic. That a viscous colloidal fluid like protoplasm would probably undergo marked viscosity changes was obvious to biologists long ago. They tried to associate many activities of protoplasm with changes in its consistency. In spite of the numerous theories that were proposed, actual attempts at measuring viscosity have until recently been almost totally lacking. Without doubt, if marked changes in protoplasmic viscosity occur, they must play an important part in the mechanics of many vital processes. There is now actual evidence to show that great viscosity changes do occur in protoplasm.

In addition to the results obtained on marine ova, Seifriz ('18, '20) and Leblond ('19) have shown viscosity changes in plant protoplasm and Bayliss ('20) has shown similar changes in *Amoeba*. Various methods have been used. The gravity method was employed by Heilbronn ('14) and later by F. and G. Weber ('17). Leblond and Bayliss used the presence of Brownian movement as a criterion of viscosity.

All of the methods are not equally reliable. To my mind the microdissection method particularly is open to question. Kite ('13) was the originator of the method. According to Kite, all egg protoplasm was a highly viscous gel which scarcely varied its consistency. After my demonstration of the fluidity of unfertilized sea-urchin egg protoplasm and the change to a gel after fertilization ('15), Chambers was able to confirm these facts with microdissection. At first I was inclined to consider Chambers' work as a valuable support to my views of protoplasmic viscosity change. In my 1920 paper this point of view is brought out. But the recent publications of the microdissectionists have made me doubt the value of the method. Compare, for example, recent statements of Chambers and Seifriz. Chambers ('19) says: "The time of appearance of the amphiaster until completion of cleavage lasts from 10 to

15 minutes. The *increased*⁵ viscosity of the egg during this amphiaser stage could be more easily demonstrated by the needle in the eggs of *Echinarachnius* and *Cerebratulus* than in those of *Arbacia*.

"After completion of the cleavage process, there are indications that the firmness of the cytoplasm persists in the two blastomeres while they are still more or less spherical."

These remarks of Chambers apparently apply to the sea-urchin egg. Working on the same egg apparently without any knowledge of Chambers' 1919 paper, to which he does not refer, Seifriz ('20) finds: "With the first appearance of the amphiasers there is a pronounced *decrease*⁵ in viscosity of the central region of the cell, and this condition is maintained throughout the intermediate stages of divisions (from middle prophase to late anaphase)." Later he says, "with the completion of division we have in each daughter cell of the embryo a general protoplasmic consistency identical with that of the egg before fertilization."

Thus Chambers considers the amphiaser stage to involve increased viscosity and Seifriz regards it as involving a pronounced decrease in viscosity (at least of the central region). And whereas, after the first cleavage, Chambers finds high viscosity, Seifriz finds a viscosity identical with that of the unfertilized egg. It might be thought that the difference is due to a difference in the capability of the two investigators, but this does not appear to be the case. For whereas Seifriz is wholly wrong in stating that the viscosity just after cleavage is no greater than in the unfertilized egg, Chambers is equally wrong in stating that the amphiaser stage is a stage of high viscosity. Evidently the difficulty lies primarily with the method. No doubt it is too subjective. Apparently all evidence as to protoplasmic viscosity gained from microdissection should for the present at least be regarded as merely suggestive.

In this paper it has been shown that during mitosis viscosity changes occur in *Cumingia* and *Nereis* which are similar to those

⁵ Italics mine.

occurring in the sea-urchin egg. The latter perhaps presents a somewhat simpler case, for the first cleavage division does not immediately follow the maturation divisions. The viscosity changes that occur in the sea-urchin egg are probably typical of mitosis in general. There is a marked viscosity increase in early prophase, then a decrease, and finally an increase just before the cell divides. This final viscosity increase in the sea-urchin egg continues as the early prophase viscosity increase of the second cleavage division. In *Cumingia* and *Nereis* the maturation divisions are related to each other and to the first cleavage division as the first cleavage division is related to the second in the sea-urchin egg. Apparently whenever two mitoses follow each other in rapid succession, the viscosity increase at the conclusion of the first provides the essential viscosity increase for the beginning of the second.

In the various eggs studied the viscosity changes are apparently not of the same magnitude. In *Nereis* they are not as great as in the other eggs. This is readily understood in the light of the fact that the *Nereis* egg is considerably larger and its mitotic spindle forms a much smaller fraction of the material of the egg.

The magnitude of the changes has been determined only for *Cumingia*. In this egg the viscosity rises from an arbitrary value of 2 or 3 to a value of 16, and then drops to about its original value. These changes are very rapid and the increase in viscosity just mentioned occurs within a minute or two. Such a sharp and sudden viscosity increase is almost conclusive evidence that a gelation or coagulation has occurred within the egg. This does not mean that the entire protoplasm has gelled. Presumably only a part is affected. It seems likely that were the entire protoplasmic mass of the egg to gel, the viscosity increase would be greater. It is more logical to assume, however, that only that portion of the cytoplasm which is concerned with spindle formation undergoes gelation. This is borne out by the fact that in the *Nereis* egg, in which the spindle forms a smaller fraction of the egg material, the viscosity change is not so great.

The mechanism of viscosity increase and decrease or gelation and solation within the cell is not yet understood. It might be

thought that gelation was due to an abstraction of water and solation to an addition of water. In an earlier paper some evidence for the first view was presented. If solation were due to an addition of water, it would naturally be expected that the breakdown of the nucleus during mitosis would be the primary cause of solation. This is, however, not the case in *Cumingia*. In the curve shown in figure 1, after the second polar body has formed the viscosity remains high for the formation of the cleavage spindle. The viscosity then drops, but this drop occurs not after the breakdown of the pronuclei, but before the pronuclei have shown any visible indication of breakdown. Thus the return of the protoplasm to a more fluid state is apparently not due to a passage of water from nucleus to cytoplasm. As far as can be judged from the published figures of Morgan ('10), Jordan ('10), the time of liquefaction is approximately simultaneous with the appearance of the fully formed cleavage spindle. The above authors show the spindle appearing at a time when the pronuclei have grown to a large size. It is at this time that the drop in viscosity occurs. Further evidence that the liquefaction of the protoplasm is not due to a breakdown of the nucleus is indicated in *Cumingia* by the fact that in this egg the protoplasm becomes more fluid immediately after the second maturation spindle is formed. In this case no nuclear breakdown occurs.

It appears that whenever a spindle forms there is a return to a fluid condition. It is as though the spindle were coagulated out of the cytoplasm. Thus the curve of viscosity at the time of spindle formation is like the viscosity curve of a coagulating albumen solution. As coagulation occurs in such a solution the viscosity suddenly increases greatly, after coagulation has been completed there is a sharp drop in viscosity. The first part of the *Cumingia* viscosity curve resembles the curve of a coagulating albumen solution given in Dunstan and Thole's 'Viscosity of liquids' (fig. 9). Of course, this resemblance may be superficial. At present, however, the idea that the spindle coagulates out probably offers the best interpretation of the viscosity changes which precede and follow spindle formation.

SUMMARY

1. The centrifuge method of measuring protoplasmic viscosity is a perfectly sound method from a physical standpoint. With it quantitative data may be obtained.

2. The velocity of granular movement under the influence of centrifugal force was taken as a measure of the viscosity.

3. The viscosity of *Cumingia* egg protoplasm was plotted for the period between fertilization and first cleavage. The data may be presented in the form of a curve (fig. 1).

4. In both maturation divisions and cleavage, appearance of the spindle is always preceded by a sharp viscosity increase and followed by a sharp viscosity decrease.

5. At the conclusion of mitosis, within a minute or two before the division of the cell is completed, a sharp increase in viscosity occurs.

6. When two mitoses follow each other, the concluding viscosity increase of the first mitosis becomes the initial viscosity increase of the second.

7. In *Nereis*, the viscosity changes between fertilization and cleavage are similar to those found in *Cumingia*. Apparently these changes are not as marked as in *Cumingia*, a fact which is correlated with the larger size of the *Nereis* egg and the relatively small size of its spindle.

8. The discussion includes a critique of the microdissection method of viscosity measurement as well as certain interpretative considerations.

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Resumen por el autor, Gary N. Calkins.

Uroleptus mobilis Engelm.

IV. Efectos del seccionamiento durante la conjugación.

Estos experimentos fueron emprendidos con el fin de averiguar lo que sucede cuando se cortan los individuos de *Uroleptus mobilis* conjugados antes de haber tenido lugar el intercambio nuclear. Los pares unidos fueron cortados con un escalpelo a través del ápice de la. Uno de los individuos fué fijado y teñido para determinar el estado de la conjugación en el momento de cortar la pareja, y el otro se dejó vivo en medio de cultivo fresco. En treinta operaciones llevadas a cabo con éxito todos los fragmentos regeneraron, experimentando los procesos de reorganización que siguen a la conjugación normal, cuyos últimos estados son visibles en el organismo viviente. Estos procesos tienen lugar sin relación alguna con el estado de la conjugación en que se encontraban los individuos al cortarlos. Siete de estos fragmentos completaron el ciclo vital con todos los rasgos característicos de los exconjugantes normales, incluso la reorganización, rejuvenecimiento, vitalidad declinante, enquistamiento, conjugación y muerte a causa de la edad avanzada. Los individuos que se han conjugado recientemente y se han separado mediante sacudidas experimentan los mismos procesos de reorganización. Del mismo modo que en los exconjugantes normales, los fragmentos cortados producen progenie cuya vitalidad depende de la edad de los padres durante el momento de la conjugación, pero en todos los casos menos en uno, esta vitalidad fué más inferior que la del exconjugante normal seleccionado como tipo de comparación, y en todo caso más baja que la de los padres. Los fenómenos de la maduración, reorganización y rejuvenescencia sin anfigamia representan una serie de todos o ninguno de los procesos producidos e iniciados con el contacto de los dos individuos durante la conjugación.

UROLEPTUS MOBILIS ENGELM.

IV. EFFECT OF CUTTING DURING CONJUGATION

GARY N. CALKINS

TEN FIGURES

In the second of these studies¹ it was demonstrated that conjugation brings about a renewal of vitality in protoplasm in which the vital activities have become reduced, or even nearly exhausted, by physiological usury. It was also shown that a similar renewal of vitality follows the reorganization which takes place asexually during encystment, indicating, as Woodruff and Erdmann have shown for *Paramecium*, that nuclear fusion or fertilization in *Uroleptus* is unnecessary in the phenomenon of rejuvenescence.

A bit of protoplasm possessing only sufficient vitality to enable it to divide once in forty days unites in conjugation with a similarly weakened and closely related bit of protoplasm; the ex-conjugant from this pairing possesses a restored vitality which enables it to divide at the rate of seventy-two times in the same forty days. As a unit mass of protoplasm or individually, the ex-conjugant is the same as before conjugation, but something has occurred in its organic make-up to bring about the change. In the first of these studies² the cytology of conjugation was fully worked out, and it was found that, as with ciliates generally, the only recognizable physical changes have to do with the nuclear complex. Not only are all of the eight macronuclei absorbed in the cytoplasm but in addition, not less than seven-eighths of the micronuclear material is likewise absorbed.

A similar absorption of nuclear substance characterizes the process of asexual reorganization accompanying encystment, and

¹ Jour. Exp. Zool., vol. 29, 1919.

² Jour. Exp. Zool., vol. 27, 1919.

the increased vitality of the individual recovered from the cyst indicates that nuclear interchange or amphimixis is not directly associated with rejuvenescence. Owing to the admixture of relatively large quantities of nuclear material, the cytoplasm of both the ex-conjugant and of the individual recovered from the cyst must be different in make-up from these cytoplasm prior to conjugation or encystment. The new micronucleus likewise has undergone some change expressed by amphimixis with conjugation, or, with encystment, some unidentified change which may be vaguely characterized but not interpreted by the mystical word 'purified.'

To test this matter of rejuvenescence without amphimixis further, and to eliminate the long period of rest necessary for reorganization by encystment, a series of experiments was undertaken in which the animals were allowed to unite in conjugation, after which they were separated by cutting with a scalpel prior to nuclear interchange. Controls for each experiment were: 1) one fixed and stained individual from each pair cut to determine the stage in maturation of the pair when cut; 2) the isolation culture series from which the conjugants were obtained, but in which conjugation was not permitted; 3) normal ex-conjugants from the same epidemic of conjugations and having the same ancestry as the cut individuals.

METHOD

A series of *Uroleptus* is selected in which vitality is sufficiently reduced to permit of unmistakable evidence of rejuvenescence. Conjugation tests are repeated until a clearly defined epidemic occurs. A pair of conjugating individuals is removed with a pipette and transferred with a minimum of culture fluid to a clean culture dish. The pair is then cut under a binocular microscope with a fine scalpel. A dozen pairs may be cut before a successful operation in the desired plane yields an individual suitable for the experiment. *Uroleptus*, fortunately, is a most satisfactory animal for this work, for its protoplasm is resistant enough to be cut in any plane without disintegration and all cut fragments regenerate perfectly. Furthermore, its method of

fusion in conjugation offers an exceptional opportunity for cutting, for, uniting by the anterior ends, the free bodies extend outwards like the two arms of a V. A successful operation cuts the V at the apex, giving two pieces if the cut is in the long axis, or three pieces if the cut is transverse. One of the arms (and the apical piece if the cut is transverse) is immediately fixed and stained. The other arm representing the second individual of the pair is placed in fresh culture medium and maintained in the same manner as a normal ex-conjugant. The processes of nuclear change are synchronous in the two individuals, hence the permanent preparation furnishes a picture of the nuclear apparatus with which the experimental animal starts.

Not only is it difficult to cut the individuals in the desired plane, but, succeeding in this, it is difficult to carry them through the period of reorganization during which mortality is high. Many successful cuts were useless because of loss of the fixed individual. Thirty successful cuts, however, were made: of these the cultural fragment died at the end of forty-eight hours in two cases; one died at the end of five days; one at the end of six days; three in seven days, one in eight, two in nine, and three in eleven days; two in twelve, two in thirteen, and two in fourteen days, while eleven reorganized perfectly, and of these, seven completed the full life-cycle.

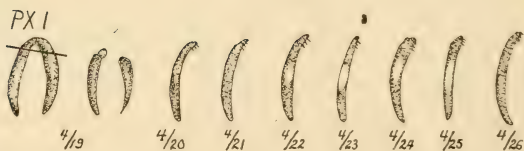
During my absence from New York in 1920 and on many other occasions the cultures were carried on by my colleague, Professor Louise H. Gregory, and I take this opportunity to express my appreciation of her careful work and my gratitude for her continued interest and help.

RESULTS

The first material to yield a successful operation was obtained from the P series in its 135th generation. Conjugations were abundant in a conjugation test on April 19, 1919. One pair was successfully cut, and the two fragments began to reorganize. One of these died, but the other completed its reorganization in seven days and was established in culture on the 27th of April 1919, as the PX1 series (fig. 1).



1



2

Fig. 1 *Uroleptus mobilis* in stage of conjugation similar to that of the pair from which series PX1 was derived. The black line indicates the plane of cutting.

Fig. 2 PX1 series: Sketches of the living fragment on successive days during reorganization after cutting, from protocols. The vesicular area indicates the relative form and size changes of the new macronucleus.

It was considered unnecessary to fix and stain one of the individuals of this pair because of the unmistakable evidence of recent union. The cut end regenerated perfectly within eight hours and the new macronucleus 'placenta' appeared in forty-eight hours as a circular vesicle in the center of the cell (fig. 2). The first division occurred on April 27th and the customary five lines of the series were established on the 29th (see fig. 9 for similar early history of any normal ex-conjugant).

The parent P series was the fifth filial generation from the original A series of *Uroleptus mobilis*, its full pedigree being: A-C-F-I-L-P. All of these ancestral series, with one exception (L) were characterized by high relative vitality as follows: A 90.9 per cent, C 97.7 per cent, F 94.1 per cent, I 89 per cent, L 74.5 per cent, and P 95 per cent.³ So far as vitality was concerned, therefore, the PX1 series had an excellent heritage.

This experimental series lived until February 14, 1920, the last division occurring on February 7th, giving a life span of 286 division days, during which it divided 281 times. The parent P series lived for 186 days after the PX1 series was started and divided 180 times, while the filial experimental series divided 190 times during these 186 days, indicating a well-marked though slight rejuvenescence. The relative vitality of the PX1 series was much lower than that of the parent, being only 71.8 per cent as against 95 per cent.

At the same time a normal ex-conjugant from the parent P series was established in culture as a control series. This, the PV series, lived for 265 division days and divided 317 times, with a relative vitality of 88.1 per cent, thus ranking in vigor with the ancestral series. It divided on the average 15.5 times in ten days during the first sixty days of life, while the parent series during the same time divided 14.2 times.

Two other pairs of conjugating *Uroleptus* from the same parent P series were cut on the same day as the PX1 series. These were the PX6 series and the PX7 series. The former underwent reorganization in seven days, while the permanent preparation shows that the individuals were in the stage of the second

³ See Studies III, Jour. Exp. Zool., vol. 29, no. 2, p. 287.

maturation division at the time when cut. The experimental individual regenerated within a few hours, but required nine days for reorganization and the first division (fig. 3). It divided on the average 14.6 times per ten days for the first sixty days of life, while the parent P series divided 14.2 times per ten days during the same calendar period, indicating, like the PX1 series, a very slight rejuvenescence. That there was rejuvenescence, however, is shown by the fact that the division rate of the P

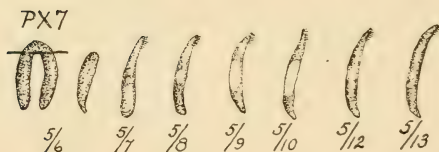


Fig. 3 PX6 series. Sketches from the living animal on successive days during reorganization after cutting.

Fig. 4 PX7 series. Sketches of the living animal during reorganization.

series from the start of the filial series up to the time of its last division was 8.5 divisions per ten days while the rate for the filial PX6 series during the same period was 11.4 divisions. The filial series lived and divided for 160 days after the parent series had died a natural death, and finally died at the age of 376 days and 313 generations. It was more vigorous, therefore, than the companion series derived from a cut individual (PX1 series), a vigor also shown by its relative vitality of 81.3 per cent, but was less vigorous than the common parent P series and less vigorous than the normal control PV series.

The other companion experimental series, the PX7 series, was obtained from a pair of conjugating individuals in which the nuclei were in the stage of the third maturation division. The individual in culture regenerated in six hours and reorganized in seven days (fig. 4). The shorter time required for reorganization is worthy of note, as it was possibly due to the late stage of conjugation at the time of cutting. Its average division rate for the first sixty days of life was 16.7 times in ten days, while that of the parent P series was 14.2 and that of the control PV series was 15.5 times. Its division rate per ten days until the death of the parent P series was 12.6 times as against 8.5 divisions for the parent series. The PX7 series, therefore, showed increased vitality over the parent protoplasm not only during the first sixty days of its life, but also throughout the remaining life of the parent. It did not live as long as the companion experimental series, however, dying out after 219 days and 264 generations and outliving the parent by only sixty days, hence its relative vitality, 64.5 per cent, was less than that of the PX1 and the PX7 series and much less than that of the parent and that of the normal ex-conjugant control.

The progeny obtained from these individuals which were cut while conjugating were normal in every respect. Conjugation tests made from time to time gave epidemics of conjugations exactly as in cases of normal ex-conjugant series. Such conjugations were normal, and ex-conjugants obtained from them gave rise to series which lived through normal life-cycles, although the relative vitality was low in every case.

Two such normal series were taken from the PX7 series in its 183rd generation on September 13, 1919, and in its 203rd generation on October 8th. These were the AX7 and the X7A series, respectively. They lived for 165 and 125 division days, respectively, and divided 191 and 163 times. The average division rates per ten days for the first sixty days of life were 14 and 15.2, respectively, as against 9.4 divisions and 5.4 divisions per ten days for the parent PX7 series during the same periods. The relative vitalities were low, amounting only to 52.7 per cent and 42.7 per cent.

The PX7 series therefore behaved like a normal series in every way, despite the facts that the process of conjugation was interrupted at the start by cutting and that no nuclear interchange occurred. It not only passed through a normal life-cycle, but



Fig. 5 Conjugating pair in the same stage as the pair which gave rise to the X75 series. The two migrating nuclei are at the anterior end. In the pair which gave rise to the X75 series, these were removed by cutting as indicated. The fragment corresponding to the shorter right-hand individual was cultivated as the X75 series.

Fig. 6 X75 series. Sketches from the living fragment during reorganization.

gave rise to two filial series which showed characteristic renewal of vitality and passed through normal, but weak, life-cycles. This weakness, indicated by the low percentages of relative vitality, may have been due to inheritance or to the old age of the parent at the time of conjugation (see *Uroleptus* III, loc. cit., for effect of parents' age on vitality of offspring).

A second group of cutting experiments was undertaken with conjugating pairs from this PX7 series. One series only, the X75 series, was successful. This pair was cut on October 10, 1919, when the parent series was in the 203rd generation. Conjugation had progressed to the stage of pronuclei migration, but the two nuclei were removed with the apex of the V by the operation (fig. 5). The experimental individual thus had completed its maturation processes, but fertilization had been prevented by removal of the migrating pronucleus (fig. 6). Six days were required for its reorganization, and it divided for the first time on October 18th. During its first sixty days of culture it divided on the average 15.5 times per ten days, while the parent series during the same period divided only 5.4 times. This series lived for only 123 division days and divided only 181 times, hence its relative vitality was low, only 46.3 per cent.

Material for a third group of cuttings was obtained from the PV series, which had served as the normal ex-conjugant control for the first group of cutting experiments. At the time of the experiments, October 15, 1919, the PV series was in its 270th generation. It had had a vigorous ancestry and its own relative vitality was high (88.1 per cent), but it was well advanced in age when the conjugation epidemic supplying the material for this group of cutting experiments occurred. A low relative vitality of the progeny was therefore to be expected, and this expectation was realized by the life-cycle of the normal ex-conjugant control for the experimental series, the Z series. This control series divided at the rate of 15.7 times in ten days for the first sixty days, while the parent PV series during the same calendar period divided at the rate of 3.8 times per ten days. The Z series lived for 168 division days, during which it divided 226 times and had a relative vitality of 57.8 per cent.

The conjugating pairs which were cut at this period and which came from the same material as the Z series gave rise to the VX1 series, the VX2 series, the VX3 series, and the XZ2 series. The conjugating pairs which gave rise to two of these series (VX1 and VX2 series) were in the parachute stage of the

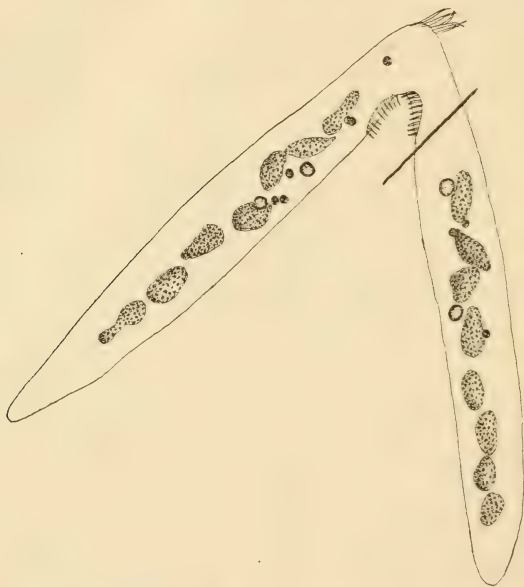


Fig. 7 Conjugating pair in the same stage as that which gave rise to the VX3 series. Both pronuclei are present, but are not yet fused in either individual. For the VX3 series the pair was cut as indicated, the left individual being cultivated.

first maturation division at the time of cutting (cf. fig. 1). A third pair giving rise to the VX3 series was cut during a late stage of conjugation and after the interchange of pronuclei but prior to the fusion of the pronuclei (fig. 7). In this case, therefore, conjugation was completed so far as the nuclear make-up was concerned. The XZ2 series, finally, came from an individual ex-

conjugant which was cut immediately after separation and before the process of reorganization had gone very far.

The cut fragment which gave rise to the VX1 series reorganized and divided by the end of the eighth day after cutting, and the



Fig. 8 Sketches of fragments which gave rise to series VX1, VX2, VX3, and XZ2, from the living organisms during reorganization.

five lines of the series were established on the 24th of October (fig. 8). During its first sixty days of life the series divided at the rate of 14.5 times per ten days, the control ex-conjugant Z series and the parent PV series dividing 15.7 times and 3.8 times, re-

spectively, per ten days during the same calendar period. It lived for 135 division days and for 192 divisions and had a relative vitality of 51.5 per cent.

The cut fragment giving rise to the VX2 series required eight days for complete reorganization and divided for the first time on the ninth day after the operation (fig. 8). The five lines of the series were established on October 26th, and during the first sixty days each divided at the rate of 15 times per ten days, the control Z series and the parent PV series dividing as stated above. This experimental series lived for 101 division days, during which it passed through 120 generations. The relative vitality was the lowest of all the experimental series, being only 31.8 per cent.

The VX3 series was derived from an individual cut during a late stage of conjugation after completion of the maturation processes and after nuclear interchange (figs. 7 and 8). The five lines were established on October 26th, and the average division rate for the first sixty days was 16 times per ten days, while the normal ex-conjugant control and the parent PV series divided at the rates of 15.7 and 3.8 times per ten days during the same period. This experimental series lived for 140 division days and 202 generations and its relative vitality was 51.3 per cent.

The XZ2 series, finally, was derived from an individual which had just completed conjugation and had begun to reorganize. The anterior end, including the peristome, was removed by cutting. Reorganization was completed in five days, and the five lines were established on the seventh day after cutting (fig. 8). The division rate per ten days for the first sixty days was 15.6 divisions, or the same as that of the normal ex-conjugant control Z series (15.7). This experimental series lived for 163 division days and for 202 generations and had a relative vitality of 52.2 per cent.

For purposes of reference and comparison, the above experimental results are summarized in tabular form in table 1.

The curve of the life-cycle of each of these experimental series agrees with the typical normal curve as plotted for successive ten-day periods. The initial division rate is high (first sixty days) and gradually decreases to nothing at the end of the life-history.

Finally, a word as to the effect of cutting individuals which are ready for conjugation. Selecting a conjugation test in which conjugation was just beginning, a number of individuals were removed and cut. Some of these were preparing for fusion, as indicated by characteristic maneuvers, and undoubtedly would have conjugated had they been left together. Forty-three individuals were successfully cut through the anterior end, after which they were treated in the same manner as cut conjugants. All regenerated, but not one showed the slightest evidence of reorganization, as shown by the formation and development of the macronuclear vesicle. Traumatic conditions were ineffective in starting up the series of changes characteristic of conjugation.

DISCUSSION

Although there is nothing in the literature nor in the history of *Uroleptus* to furnish a basis for specific expectations of any kind, the behavior of the cut conjugants was entirely unexpected. It was highly surprising to find that the course of events followed by the cut fragments was exactly the same as that of a normal ex-conjugant, despite the fact that no interchange of nuclei had taken place, and protoplasmic fusion had lasted for only a fraction of the usual time. The results indicate an 'all or none' reaction of the cells following upon fusion of two individuals at the anterior ends and involving the entire sequence of maturation phenomena.

The full history of the nuclei during conjugation and during the period of reorganization following conjugation was described in the first of these studies on *Uroleptus*.⁴ The conjugating individuals separate shortly after nuclear interchange and fusion and the subsequent reorganization processes take place in the separated ex-conjugants. The latter processes require from five to six days for completion, and the general course of events may be followed from day to day by characteristic changes of the newly formed macronucleus, which is easily visible in the living animal. Twenty-four hours after separation, this new

⁴ Calkins, *Uroleptus mobilis*, I, loc. cit.

nucleus appears as a spherical vesicular space in the center of the ex-conjugant. At forty-eight hours the vesicle is elongate or ellipsoidal, and it continues to enlarge each day until on the fourth day it becomes large enough to make up fully one-third or even one-half of the volume of the cell. Usually, on the fifth day, it condenses into a relatively small macronucleus with dense chromatin contents, and becomes invisible in the living organism (fig. 9). The nucleus is now ready for its first division, and this, with division of the cell, usually occurs on the sixth or seventh day.

Reorganization after conjugation thus requires a definite period of time during which there is no reproduction and during which characteristic morphological structures are visible in the

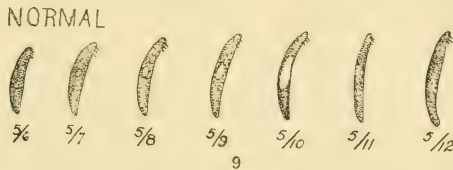


Fig. 9 Reorganization stages of a normal ex-conjugant from a living individual on successive days.

living cell. The latter is an important fact in connection with these cutting experiments.

Many of the individuals that were isolated after conjugation cutting lived for a longer or shorter time during the reorganization processes, but died without dividing. Thirty successful operations yielded only eleven individuals which completed the reorganization processes, and four of these died out shortly after the five lines of each series were established, the other seven continued to live, and their histories have been given above.

In the majority of cases the conjugating pairs were cut in such a manner that the anterior structures of the individual cells, including mouth, peristome, adoral zone of membranelles, and frontal cirri, were removed. In all cases these were regenerated within a few hours, so that the processes of reorganiza-

tion invariably took place in structurally perfect individuals. These reorganization processes without exception followed the same sequence as that outlined above for the normal ex-conjugant.

Not only reorganization processes, but the entire life-cycles which these mutilated conjugating individuals passed through showed all of the phenomena characteristic of normal life-cycles and ended in natural death. Thus previous results have shown that conjugation restores vital activities to an optimum; so here, if the individual which started the VX1 series had not conjugated it would have been able to divide on the average 3.5 times per ten days during the remainder of its life-cycle, but it actually divided 14.8 times per ten days during the same period, despite the fact that it had been cut during conjugation. The extent to which vitality had been restored in this protoplasm, therefore, is represented by 11.3 divisions per ten days. Similarly with the other experimental series derived from this same parental protoplasm, all might have divided at the rate of 3.5 times per ten days if they had not conjugated, but, having conjugated, they divided at the rates of 14.6 (VX2 series), 15.5 (VX3 series), and 15.9 (XZ2 series) times per ten days during the same calendar period, and the extent of vitality renewal is indicated by 11.1, 12, and 12.4 divisions, respectively, per ten days.

The other experimental series, PX1, PX6, PX7, and X75, showed similar results, but the extent of rejuvenescence was less—a fact illustrating another phenomenon which was described in the second and third of these studies. In the latter especially it was shown that the vitality of normal ex-conjugant series varies between rather wide extremes, and that series with low vitality came from parents which were in the weakened condition due to old age at the time of conjugation.

In order to obtain a standard measure of vitality of an entire series of *Uroleptus mobilis*, an ideal life-cycle, based upon experimental results since the first isolation of the original individual, was formulated. Such an ideal cycle was represented by a life-history of 300 division days and 350 generations, 175 days

of 'youth' and 275 generations during the period of 'youth.' The sum of these several figures (1100) was taken as the numerical expression of the vitality of an ideal series which might be used as a measure of the relative vitality of any actual series, such relative vitality being expressed as a percentage of 1100. There is no question of absolute or exact vitality involved, but the method satisfies a real need in attempts to compare the vitality of an entire series with that of another. An illustration involving the present experiments will show the usefulness of this standard of measurement. The P series was one of the strongest normal ex-conjugant series that I have cultivated, having a relative vitality of 95 per cent of the hypothetical ideal. A filial generation was taken off when the P series was 135 generations old, or in the period of high vitality. This was the PV series which served as a normal ex-conjugant control for the first group of cut conjugant experimental series, and its relative vitality was 88.1 per cent. Another filial series was taken from the P series when the latter was 291 generations old, or well advanced in old age; this, the *a* series, had a relative vitality of 44 per cent and was noticeably weak throughout the life-cycle.

The first group of cut conjugant experimental series was derived from individuals taken from the same parent stock and at the same time as the PV series. The second and third groups were taken from parent stocks when the latter were in late stages of their life-cycles (203 and 270 generations, respectively). In relation to relative vitality, the same effects were observed in the experimental series as in the normal ex-conjugant controls; those derived from young parents were more vigorous than those derived from old (table 1), but, with the exception of the X75 series, not one of the experimental series was as vigorous as its normal ex-conjugant control—a phenomenon which may have been due to the operation and subsequent reorganization, to absence of amphimixis, or it may have been purely accidental.

In regard to rejuvenescence and relative vitality, therefore, the series derived from cut conjugants behaved like normal, ex-conjugant series. They were likewise normal in other re-

spects, for example in the power of encystment and of conjugation. Conjugation epidemics occurred in all of the experimental series and many preparations were made, but these have not yet been critically studied. Ex-conjugants from the experimental series gave rise to series in which all the characteristics of any ex-conjugant series were manifested (e.g., X7A series).

There is very little evidence to indicate that the stage of maturation during which the conjugating pair were cut had any effect upon the after results. The process of normal conjugation extends over a period of approximately twenty-four hours. Judging from the number of times a given stage is found in fixed and stained preparations, it is evident that the stages leading to the first maturation require the longest time and the stage of the second maturation division the shortest time, while the period of the third nuclear division and migration of the pronuclei is again a long one. The majority of the thirty pairs that were successfully cut were in the stage of the first maturation division and the majority of the remainder were in the final stages of conjugation. The PX1 series, the VX1 series, and the VX2 series all started from individuals which were cut while in the parachute stage of the first maturation division. The PX6 and PX7 series were derived from individuals cut in the stages of the second and third maturation divisions, respectively. The X75 series came from an individual cut during a late stage in conjugation when the two wandering pronuclei were passing each other at the anterior end. This experimental individual thus had the stationary or female pronucleus ready for the mate that never came, since both migrating pronuclei were removed by the operation of cutting. The VX3 series came from an individual cut after the wandering pronuclei had passed each other and the male and female pronuclei were almost ready to unite; that is, amphimixis was assured. The XZ2 series, finally, came from an individual which was cut immediately after separation subsequent to amphimixis.

The differences and resemblances in relative vitality and characteristics of the life-cycles of these different experimental series are too slight to justify a conclusion that cutting at different stages of conjugation had anything to do with them.

CONCLUSIONS

These experimental results enable us to view the phenomena of fertilization from still another angle. It has been demonstrated both for *Paramecium* by Woodruff and Erdmann and for *Uroleptus* that renewal of vitality or rejuvenescence may be accomplished without the union of two individuals in conjugation. The phenomena of 'endomixis' precede this renewal in *Paramecium*, and the phenomena accompanying encystment precede it in *Uroleptus*. In the present experiments we prevent union of nuclei after the two individuals have united in conjugation and obtain the same results as though conjugation had continued to the end. What is it that starts the mechanism resulting in maturation divisions and reorganization leading to renewal of vitality and a new life-cycle? For *Uroleptus* at least there is no doubt that rejuvenescence follows as a result of normal conjugation, nor is there any less doubt that it follows reorganization during encystment. Rejuvenescence, therefore, is a phenomenon or an aggregate of phenomena which may be treated independently of conjugation.

Whether there is a parallel between these results with cut conjugating *Uroleptus* and certain types of parthenogenesis in Metazoa I am not ready to state. Such a problem demands a knowledge of the cytological happenings of the cut individual prior to formation of the new macronucleus, and up to the present I have dealt only with the living fragments. These living fragments show certain definite nuclear structures and happenings, and so far as they go, they indicate that the experimental animals during reorganization do not differ from normal ex-conjugants.

In any attempt to interpret these phenomena, emphasis must be laid on the fact that cutting the cell and severing protoplasmic connections with the second individual in conjugation does not stop the succession of cellular processes after they are once started. If we cut off the anterior end of a cell which has just separated from another after conjugation, the operation does not prevent the development of a new macronucleus nor absorption of the old macronucleus, nor does it interfere with rejuvenes-

cence of the ex-conjugant nor with vitality of the series derived from it. This was the case of the XZ2 series. The same is true of an individual from which the anterior end is cut off during a stage in conjugation when both pronuclei are present in the cut individual. This was demonstrated in the case of the XV3 series. Here the protoplasmic connection between the conjugating individuals was perfect, but removing the other individual by cutting did not prevent the normal reorganization of the macronucleus and restoration of vitality, nor, probably, did it prevent the union of the pronuclei which were present when the cell was cut. In these two cases therefore so far as amphimixis is concerned, the nuclear relations were the same as in any normal ex-conjugant.

Exactly the same phenomena of nuclear reorganization, rejuvenescence, and cyclical changes occurred, however, in a conjugating individual which was cut while the two migrating pronuclei were passing each other at the anterior fused ends; these were removed by the operation as described above for the X75 series. Here there could be no amphimixis nor, indeed, any diploid number of chromosomes unless the stationary pronucleus fused with a product of the third division of one of the other micronuclei. This is a possibility, but I have no evidence for or against it. Nevertheless, the regular routine of reorganization and rejuvenescence went on as though connection with the other individual had been maintained to the end. An identical history was shown by an individual from which the anterior ends were cut while in the stages of the first and second maturation divisions and in individuals cut while in the prophase stage of the first maturation division. Removal of the protoplasm of the other individual thus has no apparent effect upon the continuation of the processes of maturation once these are started.

The problem narrows down to the questions, When do these processes start? and What starts them? The latter can be answered only by hypothesis, but the former may be further analyzed. It is conceivable that the protoplasm of an individual ready to conjugate is all set, so to speak, for the succession of

maturation stages leading to reorganization and rejuvenescence and that any stimulus will start the machinery. Experiments in cutting off the anterior ends of individuals ready to conjugate result in no reorganization processes as stated above. The shock of cutting, therefore, had no effect in starting the series of nuclear and cytoplasmic changes characteristic of conjugation. We thus limit the possible time of stimulation to some period between the sexually mature prefusion individual and the beginning changes of the micronuclei after fusion of the two individuals; that is, shortly after contact of the two conjugating cells.

In picking out a pair of conjugating individuals with a pipette it sometimes happens that the individuals have only recently come together and are not firmly fused. Such organisms enter the pipette as a pair, but upon transferring to another culture dish they emerge from the pipette as two single individuals. This is an extremely rare occurrence, however, and I have vainly tried by repeatedly spurting them from the capillary pipette to separate individuals which I had reason to believe had recently come together. Such splitting of pairs is comparatively simple with *Paramecium caudatum*, but with *Uroleptus* I have never succeeded in doing it at will. On one occasion, however, when a pair had separated in the pipette without conscious effort on my part, the two individuals were isolated in separate culture dishes and treated as were the cut conjugants. This occurred with a conjugating pair during a conjugation-epidemic in the L series which was in the 203rd generation on March 15, 1919. One individual lived for thirteen, the other for fourteen days before they died, but during these two weeks each cell passed through the different phases of nuclear reorganization exactly as though conjugation had taken place. These phases, reproduced from sketches made while the cells were alive, are illustrated in figure 10.

Although these individuals died, we have here unmistakable evidence that the entire series of maturation and reorganization processes were set in motion by the first contact of the two individuals in conjugation. They were firmly enough united to

be drawn into the pipette as a pair, but not sufficiently fused to resist the pressure in the pipette. Contact of the protoplasts was established, but persisted for only a short period.

The experiments indicate that metamorphosis of the nuclei and cell is an 'all or none' phenomenon, which once started goes through to the end of reorganization whether the companion cell is associated with it or not, and with this process of reorganization is associated the renewal of vitality of the protoplasm and a new life-cycle.



Fig. 10 Successive stages in attempted reorganization of two individuals of a pair which were accidentally separated ('split') in the pipette. One individual died on the thirteenth, the other on the fourteenth day. Sketches from the living organisms.

Columbia University
New York City, March 26, 1921.

Resumen por el autor, E. J. Lund.

Influencia experimental de la polaridad orgánica por medio de la corriente eléctrica.

1. Un internodo separado de una *Obelia commissuralis* posee y retiene una polaridad inherente, según la cual la regeneración de un hidrante tiene lugar antes en el extremo apical que en el extremo basal del internodo. 2. Los internodos apicales desarrollan hidrantes antes que los internodos de origen basal. 3. Cuando se emplea una densidad apropiada de corriente eléctrica la formación de hidrantes en los extremos de los internodos puede retardarse o inhibirse por completo cuando se colocan en frente del cátodo, mientras que, al mismo tiempo, en un tanto por ciento elevado de trozos pueden formarse hidrantes en los extremos situados en frente del ánodo. 4. Bajo las condiciones que se acaban de mencionar los estolones pueden regenerar y funcionar normalmente en los extremos dirigidos hacia el cátodo. 5. Los resultados enunciados en los párrafos 3 y 4 se obtienen en trozos, bien esté dirigido hacia el ánodo el extremo basal o el apical de un internodo; es decir, la polaridad normal inherente del internodo puede invertirse por medio de la corriente eléctrica. 6. Una corriente de una densidad que apenas puede inhibir la regeneración de los internodos basales no puede inhibir la regeneración en los apicales de la misma rama. 7. El establecimiento de una polaridad electroquímica es probablemente una condición primaria, asociada fundamentalmente con el desarrollo de la polaridad morfológica, porque el mecanismo fisiológico que determina la polaridad morfológica puede influirse y dirigirse por una corriente eléctrica de origen externo.

EXPERIMENTAL CONTROL OF ORGANIC POLARITY BY THE ELECTRIC CURRENT

I. EFFECTS OF THE ELECTRIC CURRENT ON REGENERATING INTERNODES OF *OBELIA COMMISSURALIS*

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THREE FIGURES AND THREE PLATES

One of the most general properties of living cells and organisms is the symmetry and structural polarity which the parts of the individual exhibit. This symmetrical arrangement of parts of the organism generally occurs at the very beginning of development of the individual. The nature of the complex of conditions which determines this structural polarity is one of the fundamental problems of development.

The majority of biologists would probably agree that the material basis for the structure of the cell or organism is only a result of preceding physicochemical processes, in the same sense that the formation of a precipitate is the result of a chemical or physical reaction, which in turn is always associated with an energy change. If this is correct, it follows that the energy changes or forces which determine the axial or symmetrical arrangement of parts are themselves in some way and to a greater or less degree directed forces. These forces must be assumed to have an axial or polar orientation, corresponding in degree to the axial or polar arrangement of the material affected.¹

Many different methods of experimental inquiry into this problem have been used, most of which have given suggestive

¹ An excellent statement of the problem will be found in Child, C. M., *Senescence and rejuvenescence*, pp. 199-202. University of Chicago Press.

results. These have been summarized by Morgan,² Loeb,³ Child,¹ and others, and need not be referred to at this time. However, so far as I am aware, no successful attempt has been made to study the effect of the direct electric current upon the axial orientation of organs during regeneration.

The experiments given below will, I believe, conclusively show that polarity in regenerating pieces of the stem of *Obelia commissuralis* may be completely controlled and established at will by the action of a direct electric current of proper density, provided the material used has the capacity to regenerate in the absence of the electric current.

In a previous paper on form regulation of a unicellular organism (*Bursaria*) the following facts regarding polarity and reversal of polarity in the cell were found.⁴ First, reversal of polarity often occurred in cut halves of cells undergoing regeneration, with the result that typical heteromorphic individuals arose. Second, it was found that a normal cell would occasionally transform directly into a heteromorphic individual when the former was isolated from the native wild culture medium and placed in tap-water or a medium made of a weak solution of Horlick's malted milk in tap-water. Third, it was also found that the signs for this heteropolarity in the cell could be reduced to simply a *reversed beat of the cilia, without the presence of the complicated oral apparatus and mouth*. Several intermediate degrees of this reduction of structural differentiation were observed, from two perfectly normal and fully differentiated cells attached end to end to an oval-shaped mass of protoplasm with bipolar beat of the cilia. An example of this is shown in figure 1, a, b, c. The conditions for the appearance of these heteromorphic individuals were of course obscure, but at that time I called attention to the similarity of the direction of beat of the cilia in these individuals to the direction of the ciliary beat in *Paramecium* when under the influence of the direct

² Morgan, T. H., *Regeneration*. 1901. The Macmillan Co.

³ Loeb, J., *Studies in general physiology*, part I.

⁴ Lund, E. J., *Reversibility of morphogenetic processes in Bursaria*. *Jour. Exp. Zool.*, 1917, vol. 24, p. 1.

electric current (fig. 1, d, e). These observations suggested to me the possibility that reversal of polarity in a part of a regenerating piece of cell or whole animal might be associated with

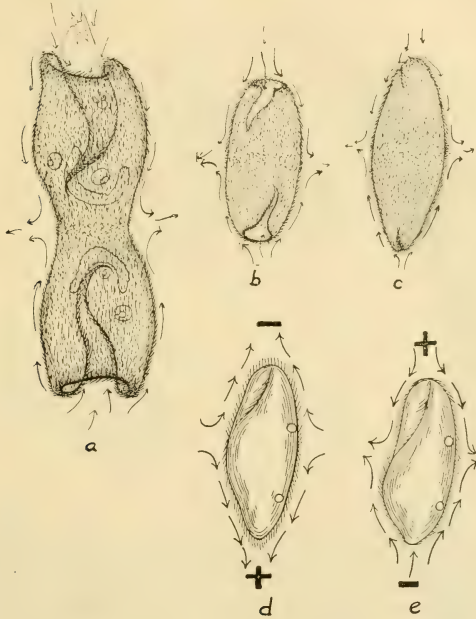


Fig. 1 *a*, *b*, and *c* are heteromorphic individuals of *Bursaria* showing different degrees of structural dedifferentiation. The chief criterion for the heteropolar condition in *c* is the beat of the cilia in opposite directions. *d* and *e* show the direction of ciliary beat in *Paramecium* in a direct electric current of proper density. *c* and *e* correspond to one another in direction of their ciliary beat. *d* and *e* might be compared respectively, to heteromorphic 'tails' and 'heads.'

a reversal of electrical differences of potential, such as those discovered in *Tubularia*, *Pennaria*, and *Campanularia* by Mathews.⁵

⁵ Mathews, A. P., Electrical polarity in the hydroids. *Am. Jour. Physiol.*, 1903, vol. 8, p. 294.

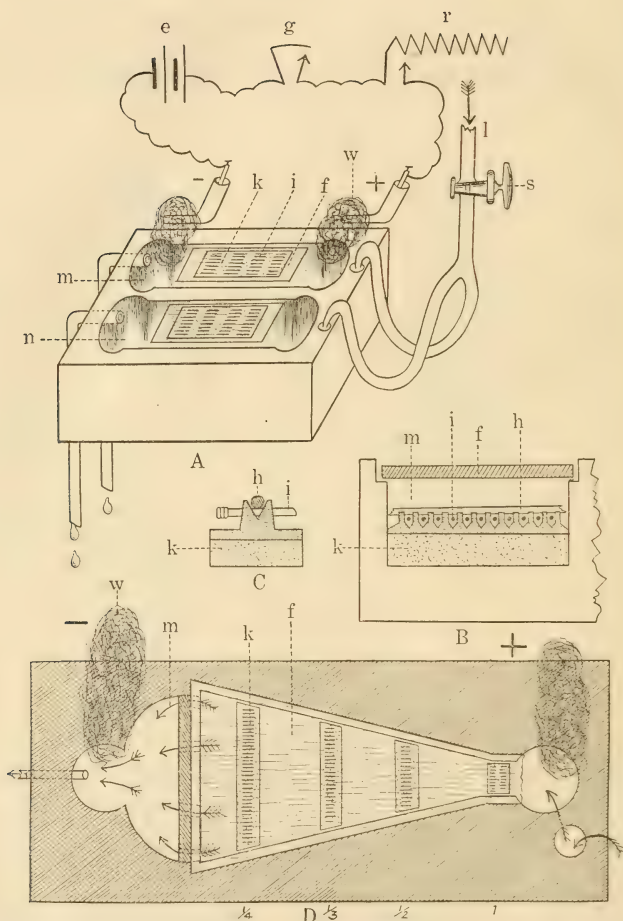


Fig. 2 shows the method for supplying satisfactory conditions for regeneration. The paraffin bath, A, has two identical troughs, *m* and *n*, through which a stream of sea-water flows from *l*, the rate of flow of which is regulated by the stopcock, *s*. An electric current from storage cells, *e*, is passed through *m*, while *n* is used for the control. The cotton bridge, *w*, acts as a siphon to prevent products of

Therefore, it ought to be possible to influence the polar character of the differentiation in regenerating structures by properly orienting such material in an electric current of proper density. This did prove to be possible, as the experiments below will show.

METHOD

The procedure described below was adopted after several attempts to obtain satisfactory experimental conditions in respect to the following: 1) Density of the electric current. 2) Aeration of the sea-water. 3) Definite orientation of the regenerating pieces of stem which were generally not more than 3 or 4 mm. in length. 4) An arrangement of the electrodes so as to prevent all possibility of products of electrolysis at the electrodes from coming in contact with the regenerating pieces. Figure 2A shows the arrangement. The current was obtained from storage and dry cells giving about twenty volts. The milliammeter, g , and resistance, r , were placed in series. The non-polarizable electrodes were connected to the troughs, as shown in A, by means of cotton dipping into the stream of sea-water which was constantly flowing through the trough from 1. To prevent zinc sulphate from diffusing into the trough, the cotton connections were adjusted to act as siphons. A continuous stream of water flowed from the trough through the cotton. Electrolysis at the electrodes results in a change of resistance and therefore variation in current intensity. To overcome this difficulty, the adjustable resistance, r , was introduced. The troughs were made from blocks of hard paraffin

electrolysis from entering m from the non-polarizable electrodes. A milliammeter, g , and adjustable resistance, r , are placed in series. The internodes, i , are held in position on the cork plate, k , by the glass rod, h , shown in more detail in B and C. The glass plate, f , covers the trough and thus maintains a constant cross-section of the conducting water column. In B is shown a cross section of one of the troughs, in B the capillary glass rod, h , is placed over internodes, i , to hold them in position, details of which are shown in C. A surface view of a gradient trough for obtaining different current densities in the same experiment is shown in D. The arrows indicate the direction of the water current. The positions of the cork strips, k , are such that the current densities at these levels in the trough are as $1 : \frac{1}{2} : \frac{1}{3} : \frac{1}{4}$.

hollowed out to meet the needs in various experiments. The sea-water was adjusted by means of the stopcock, *s*, to flow at a constant and equal rate through the two troughs, *m* and *n*. The pieces of internode, *i*, were fixed in position on a cork plate, *k*, under glass rods, *h*. The troughs were then covered with a glass plate to make the area of cross-section of trough constant. A cross-section of the trough is shown in B. The pieces of internode, *i*, were held in position by notches cut in the cork, *k*, and a piece of capillary glass rod, *h*. C shows the arrangement for holding the internode in a definite position.

In order to speak of the effects of different intensities of the electric current, its density should be known. The unit of density, δ , is usually taken to be 1/1000 of a milliampere per square millimeter of cross-section of the conductor. The current density in the trough would therefore be determined by measuring the area of the cross-section of the column of sea-water, *m*, in B, neglecting the conductance of the internodes. The conductance of the cork plate may be practically reduced to zero by boiling in soft paraffin. Since the area of the cross-section of *m* is a constant, the current density at that section will be constant throughout an experiment, other conditions remaining the same. The electrical energy flowing through the internodes depends, of course, upon the relative specific conductance of the internode and the sea-water, and therefore may conceivably vary from time to time with the progress of regenerative changes in the internode, all other conditions being the same. As a matter of fact, under the conditions of the experiments and for the purposes of the more important conclusions in the present paper, it is not necessary to know the absolute current density.

For the purpose of comparing the effects of different current densities upon polarity and regeneration, a gradient trough was constructed as shown in D. The only difference between this one and that shown in A is that the width of the column of water under the glass plate, *f*, was made greater toward one end. Each of the cork strips holding the rows of internodes were placed in such a position as to give densities of the electric current in the proportion $1: \frac{1}{2}: \frac{1}{3}: \frac{1}{4}$. The arrangement in A

provides for a control in n identical with m except for the absence of an electric current. The advantage of the gradient trough consists in increasing the possibilities of 'catching' an appropriate current density which is neither too low nor too high.

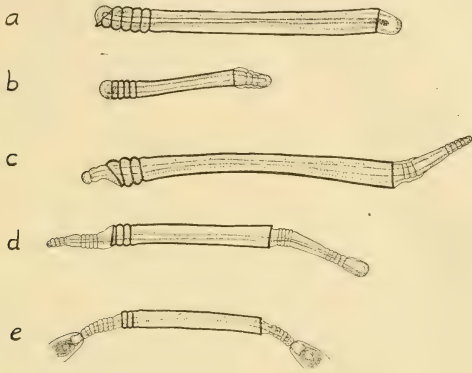


Fig. 3 Selected stages in the process of regeneration in internodes of *Obelia* in the absence of the electric current. The basal end of internode is distinguished from the apical end by the presence of segments in the perisarc. Note that hydranth formation at apical end precedes that at the basal end.

NORMAL PROCESS OF REGENERATION OF INTERNODES OF *OBELIA COMMISSURALIS* IN ABSENCE OF THE ELECTRIC CURRENT

The usual sequence of events in regeneration of internodes of the species of *Obelia* which was used is illustrated in figure 3 in the order a, b, c, d, e. The basal end of internodes can always be identified by the presence of rings in the perisarc. These never occur at the apical end of the internode.

It will be seen that development of a hydranth after cutting occurs first at the apical end of the internode and later at the basal end. A second fact which will appear in the experiments to follow, plates 2 and 3, is that internodes from the apical region of a branch very generally produce mature hydranths earlier than internodes from basal regions of the same branch.

Rare individual exceptions to the above sequence of events occur in various experiments. These facts are therefore identical with what is well known to occur in pieces cut from the stem of *Tubularia* as shown originally by Driesch⁶ and later by Morgan,⁷ Child,¹ Hyman,⁸ and others⁹.

In vigorous material under optimal conditions for regeneration internodes of *Obelia* usually formed hydranths at both ends in 100 per cent of the pieces. The control, plate 1A, shows a typical result. Whenever stolons did form, they appeared many hours after the appearance of the hydranth of the same piece, and then only, so far as was observed, at the basal end of

⁶ Driesch, H., Studien über das Regulationsvermögen der Organismen. II. Quantitative Regulationen bei der Reparation der *Tubularia*. Arch. f. Ent. Mech., 1899, Bd. 9, S. 103.

⁷ Morgan, T. H., Hydranth formation and polarity in *Tubularia*. Jour. Exp. Zool., 1906, vol. 3, p. 501.

⁸ Hyman, L. H., The axial gradients in Hydrozoa. III. Experiments on the gradient of *Tubularia*. Biol. Bull., 1920, vol. 38, p. 353.

⁹ Differences in the time which it takes for basal and apical hydranths in such regenerating pieces of hydroid stems (*Tubularia*) to reach complete differentiation have been interpreted by some to mean that differences in rate of development or 'rate of metabolism' occur at the two regenerating ends, and that this difference in 'rate of metabolism' determines the difference in the time for complete differentiation of the hydranths. This inference, it seems to me, is doubtful in the absence of adequate evidence. For, after reading the papers by various workers on this problem, I am unable to find in any of them any clear statements or data which show that the difference in time between cutting of the pieces and maturity of the hydranths is not due to a delay in the initiation of polyp formation. In fact, one is led to a somewhat contrary conclusion when reading Morgan,⁷ page 506, who states: ". . . experiments . . . seem to show that as a rule no development, or very little, takes place for a time at the aboral end" (of pieces of *Tubularia*); again, page 509: "The stimulus once received" (by the aboral end) ". . . the development can go on simultaneously with that of the oral polyp, neither suffering retardation." It is obvious that if differences of delay in initiating the differentiation occur, then hydranth formation as such might even be equally, or more rapid at a basal than at an apical end of a piece. This is a point which needs further critical quantitative study before we can speak with assurance of differences in rates of differentiation or metabolism of basal and apical ends. This consideration as to the proper use of the term 'rate' does not, however, affect the interpretation of the results and conclusions in the present paper. We are only concerned here with the possibility of electrical control of the mechanism in the regenerating piece which determines morphological polarity.

the internode. Internodes from the extreme basal region of the colonies were not used in any of the experiments.

The above description of the process of regeneration of internodes in the absence of the electric current, shows two important facts:

1. Isolated internodes possess and retain the original inherent polarity. This polarity manifests itself in the earlier appearance of the hydranth at the apical end of the internode than at the basal end of the same internode.

2. Normally under the conditions of our observations, a hydranth forms at the basal end of the internode and not a stolon, this was generally true in 90 to 100 per cent of the internode pieces.

Now, in order to show by means of the electric current that we can control and determine the physiological polarity in an internode according to which morphological polarity will develop, it is necessary to show not only that we can cause a hydranth to appear at the basal end of the internode before the appearance of that at the apical end, but also that the original normally inherent polarity of the internode can be reversed by the electric current, so that it will, for example, produce a stolon where normally a stolon would not have appeared, namely, at the apical end of the internode.

EXPERIMENTS

For brevity the experiments reported in this paper are numbered 1, 2, and 3, without reference to the total number performed or order in which they were carried out. Only a total of eight or nine experiments were performed in the limited time available for the work. The results of the experiments not reported differ only from those given in respect to the several effects which different current densities bring about in the regeneration of the piece. If, then, we describe fully the few experiments given, we will have included all the significant effects which appeared in the total number of experiments performed. Drawings to show the relative amounts of growth in each piece of all the experiments were made at different times

after the beginning of each test, so that an approximately complete history of each piece was obtained. The drawings in plates 1, 2, and 3 are camera-lucida drawings of the pieces at the end of the experiments.

Experiment 1. Trough figure 2, A, was used. Internodes were cut at random from the different branches of a colony. The age of the internode, that is, its relative position in the stem with reference to the growing point, is indicated in part by the depth of brown color in the perisarc. Apical internodes have a more transparent perisarc than more basal pieces of the same branch. One and one-half hours after cutting, the pieces were placed in position in the notches on the cork plates *m* and *n*, without reference to whether the apical or basal end of a piece pointed toward the anode in *m*. The actual orientation of the pieces with reference to the direction of the electric current is shown in B of plate 1. Immediately after placing the pieces in position, the orientation of which was determined by chance, the current was turned on and left on until the end of the experiment, forty-seven hours after cutting and forty-five hours after turning on the current. A continual stream of fresh seawater was running through *m* and *n* during the experiment. The directions of the water current and the electric current were the same in all the experiments, as indicated in figure 2, A and D. The density of the electric current was approximately 70 δ . Temperature, $15 \pm 2^\circ\text{C}$.

Twenty hours after beginning the experiment growth was well advanced toward formation of hydranths on both ends of the internodes in the control in *n*. While in *m* the majority of the pieces only showed beginnings of growth toward the anode.

Thirty hours after the beginning of the experiment, most of the pieces in *n* had fully differentiated hydranths, while pieces in *m* showed only beginnings of hydranth buds comparable to stages *b* and *c* in figure 3.

Practically no growth had occurred on the ends toward the cathode. It was also clearly evident that younger internodes in *m* showed in general greater growth than older, more basal internodes. The pieces were killed in fixing fluid and finally

stained and mounted according to the drawings made of the pieces while in position in the trough *m*. No attempt was made to retain the original orientation of the pieces in *n*, since this was immaterial for the results of the experiment. The same procedure in handling the material was followed in all the experiments.

The final result is shown in plate 1. A is the control in *n* and B the set of internodes in *m*.

Attention is called to the following facts: 1) The electric current delayed hydranth formation on the end toward the anode for about fifteen to twenty hours when compared to control. 2) Within the duration of the experiment hydranths formed only on the ends toward the anode and in this experiment in all the pieces, while no beginning of hydranth formation nor growth occurred at the cathode end. This holds for all pieces irrespective of whether the basal or apical end of the internode was turned toward the anode. Compare this result with normal development figure 3. The hydranths formed in *m* appeared normal in form and behavior.

Such complete orientation in all pieces was not obtained if the current density was not suitable, for, as will be illustrated in the following experiments, pieces from greatly different levels in the same branch 'orient' to different densities of the electric current. This experiment was not continued long enough to indicate what finally would have happened at the ends toward the cathode in *m*.

In order to show, first, the effect of different current densities upon the regeneration process and, second, the differences which occur in regeneration of apical and basal internodes from the same branch, the gradient trough figure 2, D, was used.

Experiment 2. Four vigorously growing branches from the same colony were used. The internodes from base to tip of each branch were cut in serial order and this same order was carefully retained when they were fastened in the cork strip. No attention was paid to whether apical or basal end of the internode was pointed toward the anode. The internodes from branch number IV were placed in serial order on the cork strip

in the position indicated by a current density of 1 in figure 2, D. Camera-lucida drawings of this series at the end of the experiment are shown in plate 2, B, branch IV. The actual serial order as well as the actual orientation with reference to the electric current is shown in the drawing.

The internodes from branch number III were similarly placed in serial order in the position represented by a current density of $\frac{1}{2}$ in figure 2, D. Their serial order from base to apex of the branch and actual orientation in the electric current are similarly shown in plate 2, B, branch III. Internodes from branches, II and I were placed similarly in current densities of $\frac{1}{3}$ and $\frac{1}{4}$, respectively, and similarly shown in plate 2, A, branches II and I.

The current density at position 1 figure 2, D, varied during the experiment from about 60 δ to 100 δ , and consequently the current densities at positions $\frac{1}{2}$, $\frac{1}{3}$, and $\frac{1}{4}$ varied in precisely corresponding proportions.

Twenty-three hours after beginning the experiment the extreme apical internodes of branch IV showed only slight growth on the ends turned toward the anode. In branch III the same result was noted. Greater growth in the apical internodes and noticeable growth in basal internodes occurred in branch II. Very few showed any growth on the end turned toward the cathode, but growth was considerable throughout this series on all ends toward the anode. Branch I in current density $\frac{1}{4}$ showed the greatest growth of any of the series. The apical internodes in general showed the greatest growth in the series. No apparent general difference in this series as a whole between growth toward anode and cathode was noticeable. But it is important to note that growth on the apical end of every internode in branch I was greater than on the corresponding basal end of each piece (cf. fig. 3 above).

At thirty-four hours each piece was again drawn freehand, removed, numbered, and finally stained and mounted in the exact order and position in which they had been placed in the trough. Plate 2, A and B, shows the extent of regeneration in the different current densities. The time of appearance of hydranths in a control carried parallel to the experiment showed practically the same result as in plate 2, B, branch I.

By a careful study of these drawings the following facts appear:

1. The greater the current density the greater the inhibition of regeneration. This occurs to a lesser extent in the end which happens to be turned toward the anode than in that turned toward the cathode.

2. There is a lower current density, branch I, below which regeneration takes place in practically a normal manner. It should be noted that in branch I the regeneration at the apical end of all the internodes preceded that at the basal end; i.e., *the electric current did not overcome the inherent polarity of the pieces in branch I*. This means that with respect to the effect of the current there is something which is similar to a threshold density. Compare the orientation of *Paramecium* in an electric current.

3. Regeneration on the end toward the cathode, branches II, III, and also IV, is inhibited to a greater degree (if not completely) than regeneration on the end toward the anode, irrespective of whether the apical or basal end of the internode is pointed toward the cathode.

4. The density of the electric current necessary to inhibit (delay) regeneration in apical internodes is greater than that necessary for inhibition of regeneration in more basal internodes of the same branch. That is, the threshold density for inhibition of apical internodes is higher than for basal internodes, other conditions being the same. This statement corresponds to the fact that apical internodes in the absence of the current regenerate sooner than more basal internodes of the same branch. Branch I was practically normal in its regeneration and might as a matter of fact serve as a control for the results in branches II, III, and IV.

The result in this experiment does not differ from that in experiment I, for if we had selected internodes from the level of branches corresponding to that of the middle region of branch III for example, and left them to regenerate for forty-five hours instead of thirty-four hours then we should have expected the same results.

Experiments 1 and 2 were discontinued before it was possible to decide what regenerative processes, if any, would finally have occurred in ends toward the cathode and those turned toward the anode, which showed no regeneration. For this reason experiment 2 was repeated.

Experiment 3. The current density was slightly greater than in experiment 2. This is also clearly evident when the results in branch I, experiment 2, is compared with branch I in this experiment. The material was obtained from vigorous growing colonies on the laboratory float. Duration of the experiment was sixty-six hours, thus allowing time enough for regeneration of stolons, if these would develop in the pieces.

Observations were made at intervals throughout the experiment. At thirty-one hours the difference in growth between ends toward the anode on basal and apical internodes was very clearly shown in branches I, II, and III. A very much greater growth occurred toward the anode than toward the cathode in the same branches. Only traces of growth, if any, appeared in branch IV. The greater orienting effect of the stronger current in this experiment was plainly evident when compared to that in experiment 2 at the same interval.

At the end of sixty-six hours (plate 3, A, B), branch IV in relative current density of 1 shows practically complete inhibition at both ends of the internodes. Only two immature polyps had developed on apical ends toward the anode, which without the current would have reached full development in less than forty hours. Two undeveloped stolons appeared at ends toward the cathode.

Branch III regenerated stolons on all internodes which showed any appreciable growth on the cathode end.

Hydranths formed only on the anode ends of the pieces, and apical internodes in general developed hydranths earlier than basal internodes of the same branch. This therefore shows that, irrespective of whether the basal or apical end of the internode is turned toward the anode, stolons can develop on the end toward the cathode.

In branch II thirteen internodes formed stolons on ends toward the cathode, nine showed no growth toward the cathode, within sixty-six hours, while only one internode from the apical end formed a hydranth toward the cathode. Again no stolons formed toward the anode.

In branch I no stolons formed. Hydranths were more numerous on the anode than on the cathode side, showing a marked orienting effect of the current. However, the current was not strong enough to bring about any growth of stolons, on cathode end, instead of hydranths, within the limit of sixty-six hours.

In removing the pieces of branches II and III, most of the stolons were broken off, because of their attachment to the side of the cork plate. Hence they are not shown complete in the drawings. Pieces of cork are shown attached to some of the stolons.

The important fact which this experiment shows besides confirming all the previous statements is that *inhibition of hydranth formation at the cathode in a suitable current density does not mean that the capacity of this tissue to grow is lost, but rather that the tissue can and does differentiate into a stolon which functions normally, and this occurs irrespective of the original orientation of the internode in the current.* We have therefore fulfilled the second requirement for a proof, page 479 above, that *an electric current of proper density can reverse the inherent polarity of an internode and determine the direction of the physiological axis in the piece according to which morphological development and differentiation take place.*

It might be supposed that light played a part in determining the orientation, since growth in some hydroids shows a directive effect due to light, e.g., Eudéndrium. That light plays no part in the result is evident from the control in experiment 1, also branch I in experiment 2. Other experiments showed clearly that light did not enter into the experiment as a directive factor. The possible directive effect of the water current is ruled out for the same reasons as those for light.

Without proper test, it might be assumed that the difference in the time between the appearance of hydranths on the apical and basal internodes of the same branch, experiments 2 and 3, were due to a difference in current density on the two sides of the gradient trough, one side of the water column being thicker than the other. This objection is removed by the fact that the serial apicobasal order of the internodes of branch I, experiment 3, was purposely inverted, yet the earlier observations during the experiment showed clearly that hydranths developed earlier on apical than on basal internodes. Furthermore, in experiment 1, apical internodes showed earlier development than slightly more basal internodes, even though no serial order was maintained. Finally, measurements of the thickness of two sides of the trough showed no difference. A similar reasoning would seem to rule out contact stimulus as a determining factor.

Several experiments not as comprehensive as those presented and in which fewer internode pieces were used confirm all the observations and conclusions to be drawn from the experiments presented above. Many questions, of course, remain to be answered, some of which will be considered in following papers. The above experiments constitute, therefore, only a hopeful beginning of the analysis of the problem of polarity by means of this method. Under the conditions of the observations the following conclusions may be drawn:

SUMMARY

1. Internodes cut from *Obelia commissuralis* possess and retain an inherent polarity, according to which regeneration of a hydranth occurs at the apical end before that at the basal end of the internode.

2. Apical internodes develop hydranths earlier than internodes of basal origin.

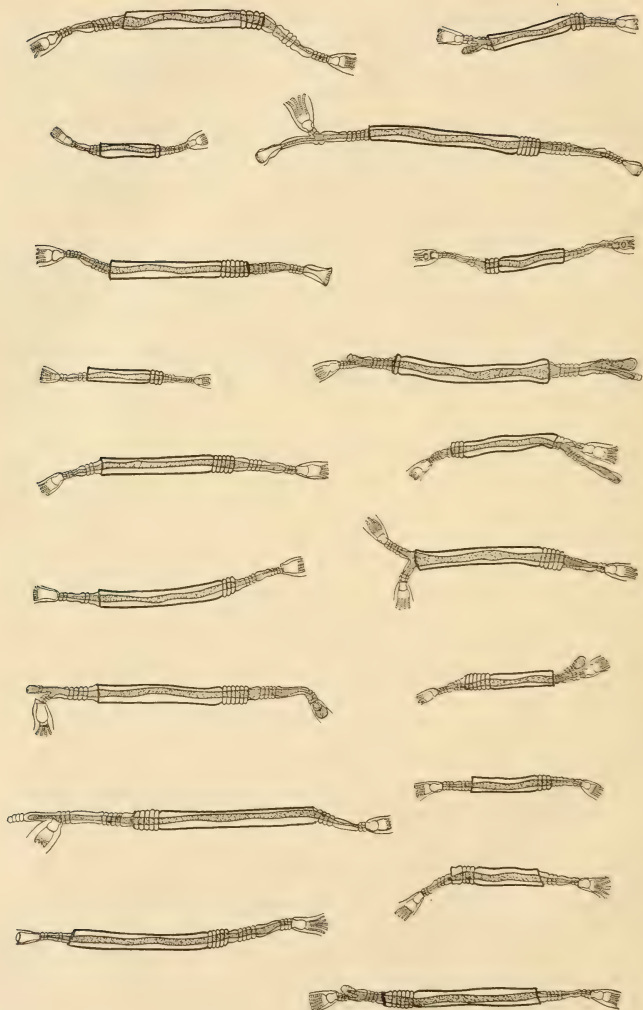
3. With an appropriate density of electric current, all hydranth formation on ends of internodes when turned toward the cathode can be delayed or entirely inhibited, while at the same time hydranths may form in a high percentage of pieces on ends pointed toward the anode.

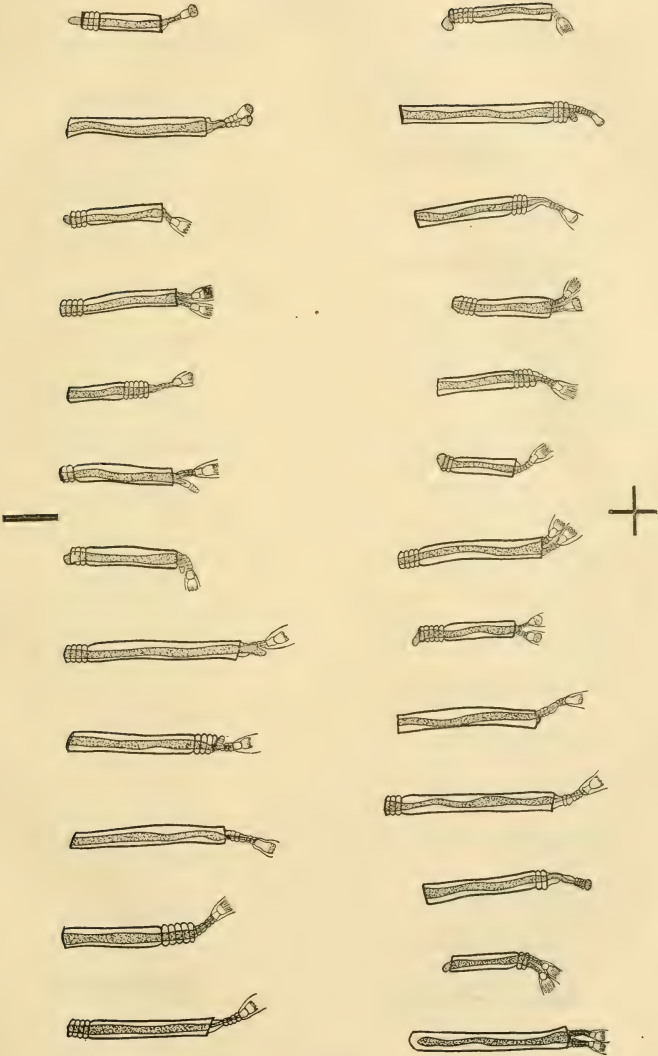
4. Under conditions in 3, stolons may regenerate and function normally on ends toward the cathode.

5. Statements under 3 and 4 hold for pieces irrespectively of whether basal or apical end of an internode is pointed toward the anode; that is, the inherent normal polarity of the internode may be reversed by means of an electric current.

6. A current density barely able to inhibit regeneration in basal internodes cannot inhibit regeneration in apical internodes of the same branch.

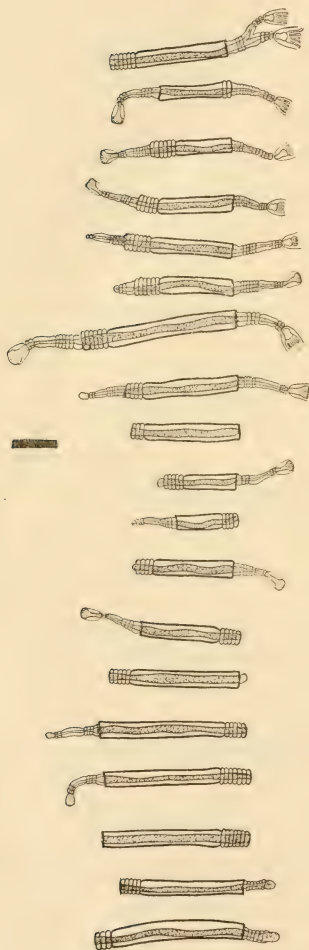
7. *The establishment of an electrochemical polarity is probably a primary, fundamentally associated condition for the development of morphological polarity, because the physiological mechanism which determines morphological polarity can be controlled and directed by an electric current of external origin.*





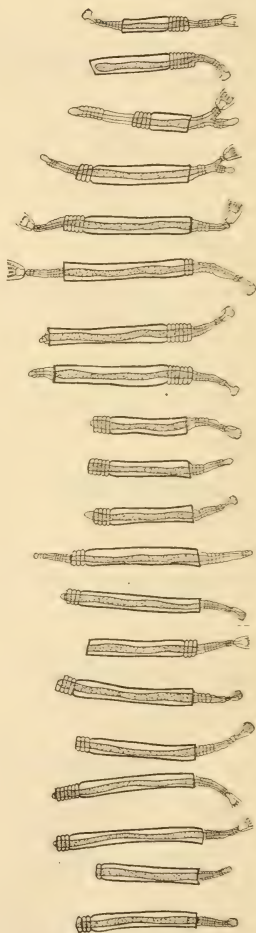
Apical

Current density $\frac{1}{4}$



Branch I

Current density $\frac{1}{8}$

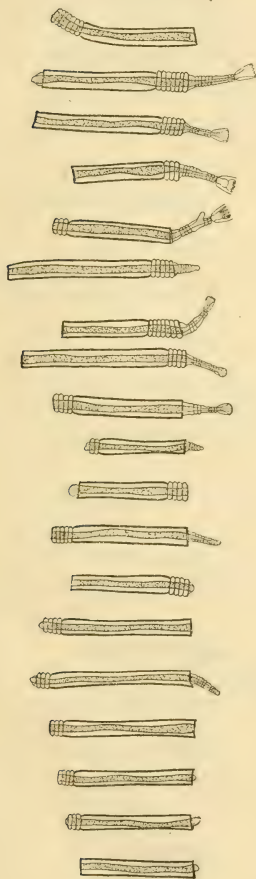


Branch II

Basal
490

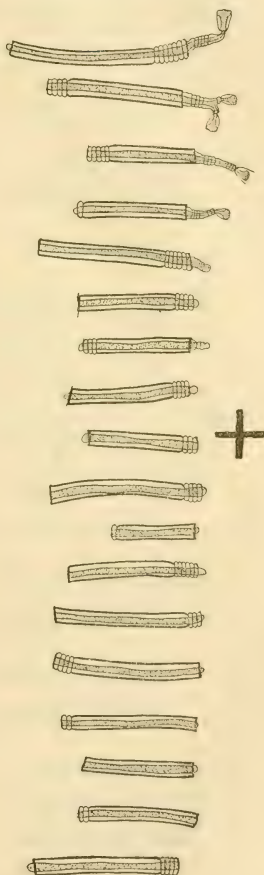
Apical

Current density $\frac{1}{2}$



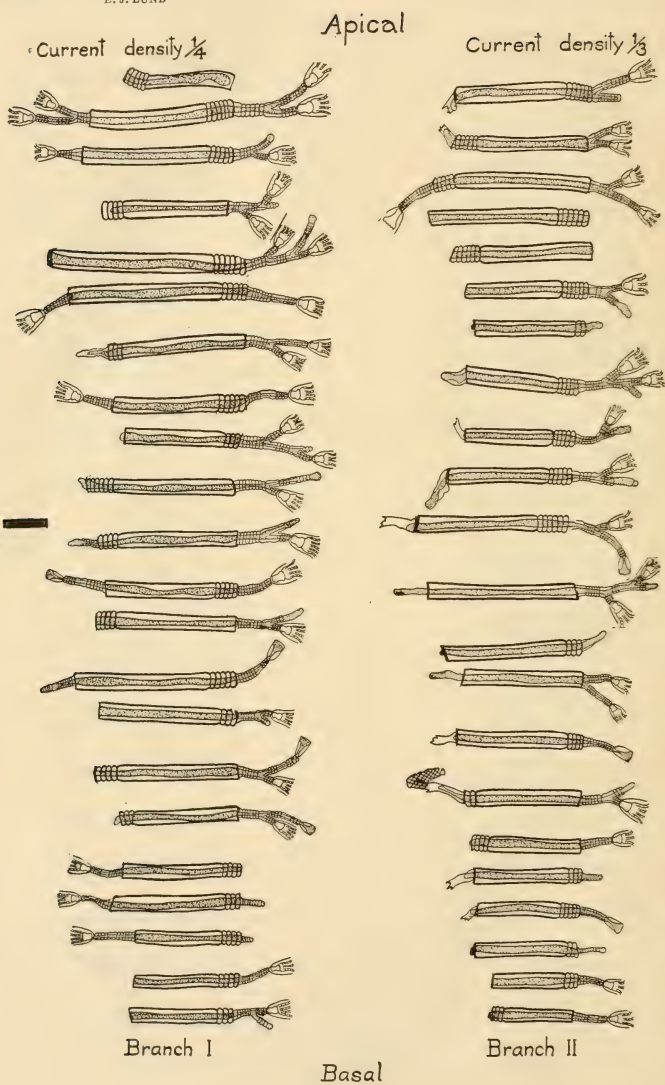
Branch III

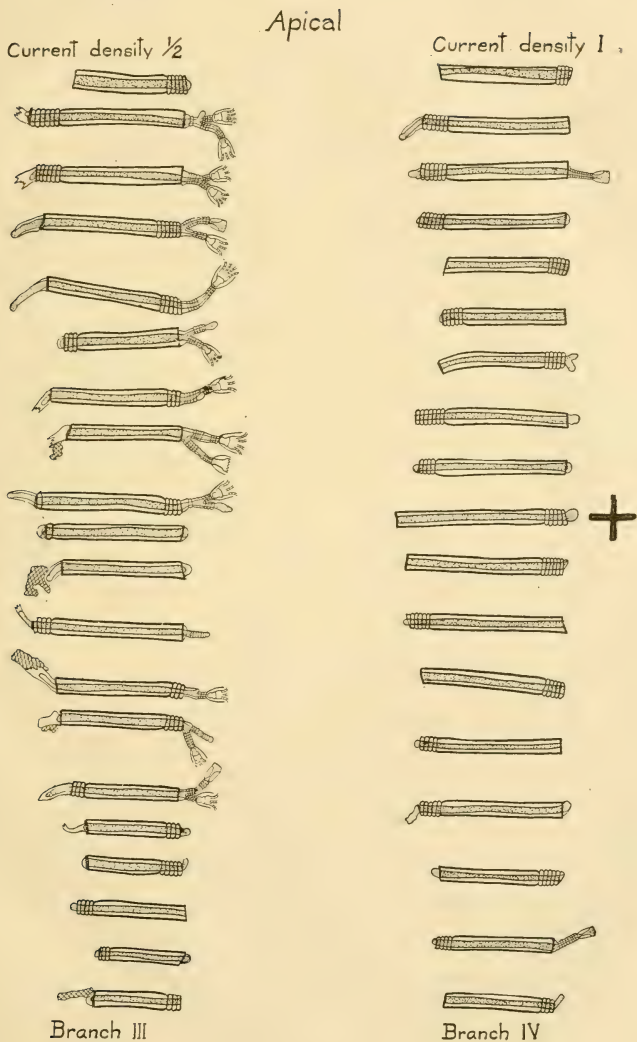
Current density I



Branch IV

Basal





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